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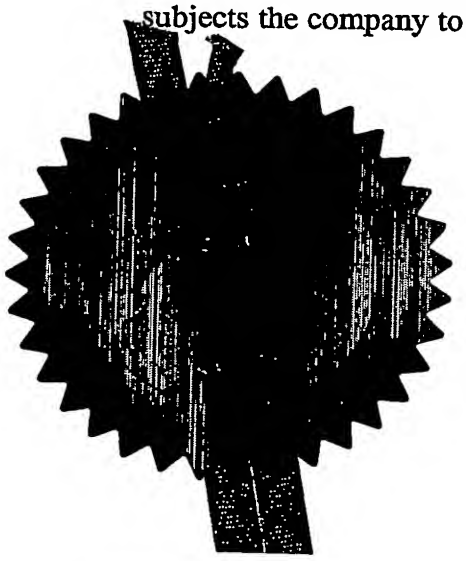

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1. Your reference GBP286912

2. Patent application number
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3 Full name, address and postcode of the or of each applicant (underline all surnames)

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Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation U.K 7676620001

4. Title of the invention Removal and Modification of the ~~Immunological~~ Immunoglobulin Constant Region Gene Cluster of a Non-Human Mammal

5. Name of your agent (if you have one)
"Address for service" in the United Kingdom to which all correspondence should be sent
(including the postcode)

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Patents ADP number (if you know it) ~~18001~~

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Country	Priority application No (if you know it)	Date of filing (day / month / year)
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Document : 900654

Removal and modification of the immunoglobulin constant region gene cluster of a non-human mammal

This invention relates to genetically modified non-human mammals, in particular to genetically modified rodents such as mice, which do not encode any endogenous immunoglobulin heavy chain constant region locus polypeptide. The invention also relates to genetically modified non-human cells, particularly embryonic stem cells, especially rodent cells such as mouse cells, which do not encode any endogenous immunoglobulin heavy chain constant region locus polypeptide. The invention also relates to genetically modified non-human cells, particularly embryonic stem cells and genetically modified non-human mammals produced therefrom, and their use in the production of non-human mammals and cells from which all the endogenous immunoglobulin heavy chain constant region genes (from Cμ to Cα) have been deleted from the genome.

This invention further relates to genetically modified non-human mammals, in particular to genetically modified rodents such as mice, in which the deletion of the endogenous immunoglobulin heavy chain constant region genes has been used to allow insertion of other genes, such as exogenous immunoglobulin genes or their segments, to secure and allow immunoglobulin heavy chain locus specific gene expression. Additionally, the invention relates to the expression of such genes produced by such mammals, and the use of such mammals or cells thereof in the production of modified immune systems with particular emphasis on production of immunoglobulins or antibodies.

Furthermore the invention relates to non-human mammals without any endogenous constant region genes bred with compatible non-human mammals capable of expressing exogenous immunoglobulin genes either as introduced rearranged entities or entities in germ line configuration and on large chromosome fragments

such as BACs, YACs and HACs (bacterial, yeast and human artificial chromosomes). The transgenes and transloci can be of human DNA.

Background to the Invention

The basic structure of an antibody (immunoglobulin) is a four polypeptide unit, made up of two heavy and two light chains held together by interchain disulphide bonds. Each chain is made up of a variable and a constant region; V_L and C_L are the generic terms for these regions on the light chain, V_H and C_H specify the variable and constant regions of the heavy chain. In a conventional antibody, each pair of heavy chains is identical as are each pair of light chains.

Immunoglobulins (Igs) are grouped into classes or isotypes (referring to the constant region) on the basis of the structure of their heavy chain constant region (C_H). In human and mouse there are five classes: IgG, IgA, IgM, IgD and IgE. The additional subclasses in human are IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2 and in mouse IgG1, IgG3, IgG2a and IgG2b. The isotype is defined by the distinct C genes (μ, δ, γ, ε, α) which are characteristic for a particular mammalian species and are present in all members of that species. However, there can be differences, usually point mutations or small changes, in immunoglobulin genes within members of a species (e.g. BALB/c mice versus C57/Bl6 mice). These differences define allotypic determinants. Allotypic determinants are found on the immunoglobulins (Igs) of some, but not all, members of a particular species (e.g. *mus musculus*). Allotypic determinants reflect genetic polymorphism of the heavy chain constant region of Igs within one species and have been used as genetic markers. Antibodies that allow recognition of isotypic determinants have been raised by injecting immunoglobulins from one species into another species (e.g. human IgM into a mouse) or between species members which allow to produce anti-allotypic antibodies (BALB/c IgM^a into C57/Bl6 which make IgM^b).

The light chain constant regions exist in isotypic forms known as kappa (κ) and lambda (λ), these light chains can potentially associate with all heavy chain

isotypes. The light chains in a given antibody are identical, so immunoglobulins have either κ or λ light chains, but not mixed light chains, unless the antibody has been artificially engineered.

Idiotypic determinants exist as a result of unique structures generated by the hypervariable subregions (or complementarity-determining regions, CDRs) in the light (L) and heavy (H) chain variable regions and have been identified by sequence variability.

Techniques have been developed for production of monoclonal antibodies using cells derived from small laboratory animals, such as mice (Köhler, G. & Milstein, C. 'Continuous cultures of fused cells secreting antibody of predefined specificity.' Nature 256, 495-497 (1975).) which first described production of monoclonal antibodies by fusing an individual antibody forming cell with a B-cell tumour cell to produce a constantly dividing clone of cells (hybridoma cell line) dedicated to making one particular antibody (monoclonal antibody).

A monoclonal antibody (mAb) has the ability to recognise a defined structure or sequence or bind to a specific epitope. Thus a monoclonal antibody can be used uniquely to identify molecules carrying the specific epitope or may be directed, alone or in conjunction with another moiety, to a specific site for diagnosis or therapy.

The use of monoclonal antibodies as therapeutic agents has been restricted by the development of an anti-immunoglobulin response in the treated subject. Because the sequence of the constant region is specific for the species from which the antibody is produced, the introduction of a foreign (xenogeneic or exogenous) antibody into the vascular system of the host can produce an immune response. When the xenogeneic antibody is introduced repeatedly, for example in the treatment of chronic diseases, it becomes impractical to administer the antibody, since an immune response is provoked and will result in eliminating the target efficiency of the administered antibody by generating extreme side effects such as anaphylactic shock. For this reason various efforts have been made to "humanise"

rodent antibodies already successfully used for therapeutic intervention and, more recently, to establish transgenic mice producing fully human antibodies (Brüggemann, M. and Taussig, M.J. (1997) Production of human antibody repertoires in transgenic mice. Curr. Opin. Biotechnol., 8, 455-458).

The production of transgenic animals has been revolutionized by techniques that allow culture of murine embryonic stem cells and the introduction of genetic modifications into these cells that can be transmitted to the mouse germline. Endogenous genes can be modified and foreign (exogenous) genes, such as human genes, can be introduced into a host to provide animal strains capable of producing novel products such as xenogeneic binding proteins, in particular immunoglobulins.

As an alternative to the use of artificial libraries, it is possible to create transgenic animals carrying human immunoglobulin loci, in germline configuration, in a genetic background in which the endogenous Ig loci are defective. Silencing of immunoglobulin genes has been carried out by gene insertion and gene removal utilising the introduction of gene targeting constructs into embryonic stem cells (Bot, A., Immunoglobulin deficient mice generated by gene targeting as models for studying the immune response. Int. Rev. Immunol., 13, 327-40,1996; Loffert et al., Early B-cell development in the mouse: insights from mutations introduced by gene targeting. Imm. Rev. 137, 135-53, 1994). In these animals it has been shown that introduced loci can replace the function of silenced genes; they are expressed and, for immunoglobulin genes, are rearranged with similar efficiency than the silenced genes.

Brüggemann et al (PNAS 1989; 86: 6709 - 6723) describe production of mice carrying a human heavy chain minilocus with unrearranged Ig variable (V), diversity (D) and joining (J) elements linked to a human heavy chain constant μ gene, which encodes the IgM immunoglobulin isotype. The foreign (human) immunoglobulin genes are inserted into the germline of the transgenic mice, with the result that the foreign insert is present in addition to the endogenous Ig genes. In these mice the foreign genes can rearrange to encode a repertoire of immunoglobulins of the IgM isotype.

The work by Brüggemann *et al (ibid)* is also described in US patent specification no. 5,545,807 which relates to a method of producing an immunoglobulin obtained from cells or body fluid of a transgenic animal which has had inserted into its germline genetic material that encodes for at least part of an immunoglobulin of foreign origin or that can rearrange to encode a repertoire of immunoglobulins. Therein, it is suggested that "it may be convenient to use a host animal that initially does not carry genetic material encoding immunoglobulin constant regions so that the resulting transgenic animal will use only the inserted foreign genetic material when producing the immunoglobulins. This can be achieved either by using a naturally occurring mutant lacking the relevant genetic material or, by artificially making mutants e.g. in cell lines ultimately to create a host from which the relevant genetic material has been removed." However, no suggestion is made as to how to provide such an IgC-deficient host animal and the teaching focuses only on insertion of human genetic material; no example is given in which the endogenous Ig heavy chain constant region is deleted.

EP 0 463 151 discloses mice in which a 2.3 kb fragment of the endogenous mouse heavy chain locus, carrying the D and J1-4 genes, is removed and replaced (by homologous recombination) with a neomycin resistance gene. The endogenous mouse IgH C genes are present in the mouse, but because the D and J genes are absent, rearrangement of the locus cannot take place and expression of the endogenous IgH C genes is blocked, preventing production of a functional message encoding an IgH C subunit.

Kitamura et al (Nature 1991; 350: 423 - 426) describe production of mice with a disrupted Cμ region. This was achieved by gene targeting, in which a 9 kb genomic fragment of Cμ and Cδ carrying a stop codon and flanked by a 5' neomycin resistance gene and a 3'HSV tk gene, was inserted into the membrane exons of Cμ using embryonic stem cells. The ES cells were used to generate chimaeric animals, which were bred to obtain mice heterozygous and then homozygous for the disrupted Cμ region. In mice homozygous for the disrupted Cμ region,

development of B cells is arrested at the stage of pre-B-cell maturation and thus they do not produce mature or antibody secreting B-cells.

There is a desire to produce genetically modified non-human mammals, e.g. rodents such as mice, in which the endogenous IgH genes have been silenced, or removed, in order to produce antibodies of foreign origin, expressed from introduced genes.

A number of approaches have been used for silencing (i.e. disruption), or removal, of single endogenous Ig genes, combined with simultaneous introduction of one exogenous human gene.

Pluschke *et al* (J. Immunol. Methods 1998; 215 (1-2): 27 - 37) used a conventional gene targeting strategy (Stief *et al.*, J. Immunol. 1994; 152: 3378) in embryonic stem cells to replace the mouse IgH constant gamma 2a (Cγ2a) gene segment with the human IgH constant gamma 1 (Cγ1); in addition to this, ES cells were generated in which the mouse IgL kappa gene segment was replaced with its human counterpart. The ES cells were used to generate chimaeric mice.

Zou *et al* (Science, 1993; 262, 1271 - 1274) describe a Cre-*lox*P recombination system that operates in mammalian cells and has been used for gene targeting experiments in the mouse to generate "clean" deletions of target genes in the germ line, as well as to inactivate genes in a conditional manner (based on regulated expression of Cre recombinase).

Cre is a 38 kDa recombinase protein from bacteriophage P1 which mediates intramolecular (excisive or inversional) and intermolecular (integrative) site specific recombination between *lox*P sites; for a review of the system refer to Sauer in Methods of Enzymology; 1993, Vol. 225, 890-900. A *lox*P site (the locus of crossing over) consists of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region. One molecule of Cre binds per inverted repeat, or two Cre molecules line up at one *lox*P site. The recombination occurs in the asymmetric spacer region. Those 8 bases are also responsible for the directionality of the site. Two *lox*P sequences in opposite orientation to each other invert the intervening

piece of DNA, two sites in direct orientation dictate excision of the intervening DNA between the sites, leaving one *lox*P site behind. This precise removal of DNA can be used to eliminate genes (gene deletion) or to activate genes. The Cre-*lox*P system can also be used to introduce genes (gene introduction). A gene flanked by two *lox*P sites in a direct orientation is referred to as a "floxed gene".

Zou *et al* (Current Biology 1994; 4: (12) 1099 - 2003) describe use of the Cre-*lox*P system in mouse embryonic stem cells to replace the mouse gene Cγ1, which encodes the constant region of the heavy chain of IgG1 antibodies, with the corresponding human gene Cγ1. A targeting construct was generated in which a *lox*P site was cloned at the 3'end of the target gene sequence (in this instance the mouse Cγ1) and, at a position 5' of the target gene, an insertion was made of (from 5' to 3') a mutant gene of interest (in this instance human Cγ1), a *lox*P site, a negative selection marker (HSV-tk) and a positive selection marker (neo^r). In the construct the *lox*P sites were in direct orientation. The targeting construct was introduced by transfection into ES cells, transformants were selected on G418 by neomycin resistance. A Cre construct was introduced into the transformed cells to achieve transient expression of Cre. Recombination, that is excision of the sequence between the two *lox*P sites (encoding HSV-tk, neo^r and the endogenous target gene mouse Cγ1), occurred only in those cells expressing Cre recombinase. The human Cγ1 sequence was situated outside the *lox*P sites and thus remained inserted within the mouse genome. Negative selection using acyclovir or gancyclovir was used to identify those cells in which the deletion had taken place, as only cells that do not express HSV-tk, i.e. those in which the endogenous mouse Cγ1 gene has also been deleted, were able to survive on those media.

Thus, Zou *et al* (1994) used the Cre-*lox*P system to introduce a human Cγ1 gene and then delete a single endogenous Ig heavy chain constant region gene, Cγ1. The exons encoding the transmembrane and cytoplasmic portions of the IgH mouse Cγ1 were not replaced by human sequences, these were retained to minimise the risk of disturbing membrane expression and signalling of the humanised IgG1 in the mouse. The introduced human Cγ1 gene was transmitted through the mouse

germline and the resulting mutant mice were crossed with mice expressing kappa light chains with a human, instead of mouse constant region. Mice homozygous for both insertions produce humanised kappa chain bearing IgG1 antibodies.

Nicholson *et al* (J Immunol. 1999; 6888 – 6906) produced mice that carry YAC based human Ig heavy and both κ and λ light chain transloci in a background in which the endogenous IgH and Igκ loci have been inactivated. Inactivation of the IgH locus was achieved using the mice described by Kitamura (1991) *supra*, Igκ expression was disrupted by insertion of a Neo cassette in the Cκ gene (Zou et al Eur J Immunol 1995, 25, 2154).

A technical problem addressed by this invention is the production of a non-human mammal, that is unable to express any of its endogenous IgH C genes and thus is immunodeficient. A particular problem addressed by the present invention is the production of a rodent, in particular a mouse, that is unable to express any of its 8 endogenous IgH C genes. To achieve this, it is necessary to generate a mutant non-human mammal, in particular a rodent such as a mouse, in which the endogenous heavy chain constant region genes are no longer present or not functionally active.

Accordingly the present invention provides a genetically modified non-human mammal or a genetically modified non-human mammalian cell characterised in that it does not comprise a nucleic acid sequence which itself encodes any endogenous immunoglobulin heavy chain constant region locus polypeptide. The invention also provides a genetically modified or transgenic mouse wherein the germ cells are free from the endogenous immunoglobulin C gene locus. The invention further provides a genetically modified transgenic mouse or the progeny thereof, wherein the somatic and germ cells are free from the endogenous immunoglobulin C gene locus.

In an aspect of the invention, the genetically modified non-human mammal or cell does not comprise a nucleic acid sequence which itself encodes any immunoglobulin heavy chain constant region locus polypeptide. In preferred genetically modified non-human mammals and cells of the invention, all

immunoglobulin heavy chain constant (IgH C) region gene sequences are absent or partially absent from the genome. Preferably each of the endogenous IgH C region genes is absent; more preferably the entire endogenous C region (from Cμ to Cα) is absent.

Herein, endogenous defined as authentic, native, not foreign and not modified by genetic engineering such as gene targeting or gene introduction.

Genetically modified non-human mammals or cells are obtainable by targeted deletion of all or essentially all endogenous IgH C gene sequences. The deletion can be of all endogenous IgH C region genes and intervening sequences (complete exon/intron removal or clean deletion) or essentially deletion of an extensive part of the endogenous IgH C region gene sequence such that expression of any of the IgH C genes is prevented. Targeted deletion can be performed by a recombination-excision process, for example by Cre-*lox*P recombination. Thus the invention further provides a genetically modified or transgenic non-human mammal as described herein or a genetically modified or transgenic non-human mammalian cell preferably an embryonic stem cell as described herein, obtainable by a site specific recombination method, preferably by a Cre-*lox*P recombination method.

In site specific recombination methods for targeted deletion, a region of nucleic acid sequence flanked by two site specific recombination sequences is excised; following excision, a single site specific recombination sequence remains within the genome. It is preferred that the site specific recombination sequence is a non-endogenous site specific recombination (NESSR) site. Several methods can be used to produce a non-human mammalian cell in which the target sequence for deletion, i.e. the endogenous IgH C locus, is flanked by NESSR sites. In one such method, NESSR sites are sequentially integrated into the genome of a non-human mammalian cell, preferably an embryonic stem cell, so that a NESSR site is first introduced to a cell and integrated at one end of the target sequence and secondly a NESSR site is introduced and integrated at the other end of the target sequence. In an alternative method, the NESSR sites are introduced simultaneously to the cell for integration at each end of the target sequence. Cells with a NESSR site at one or

other end of the target sequence can be used to produce genetically modified non-human mammals with NESSR sequences present within the genome at one or other end of the target sequence. A non-human mammal having a single NESSR site at one end of the IgH C locus target sequence can be bred with non-human mammal having a NESSR site at the other end of the IgH C locus target sequence, to produce progeny with NESSR sites flanking the target sequence. Cells with NESSR sites flanking the IgH C locus target sequence can be used to produce genetically modified non-human mammals with NESSR sequences present within the genome flanking the endogenous IgH C locus.

The present invention provides a genetically modified non-human mammal or cell having at least one non-endogenous site-specific recombination sequence present within the genome downstream of, or within the last gene of the IgH C locus and/or upstream of, or within the first gene of the IgH C locus. In a preferred embodiment two NESSR sites, preferably *lox*P sites, are integrated within the genome downstream of, or within the last gene of the IgH C locus and upstream of, or within the first gene of the IgH C locus.

An NESSR site may be present upstream at a position adjacent to the first gene of the IgH C locus and/or downstream at a position adjacent to the last gene of the IgH locus. By "adjacent to", it is meant that the NESSR site is positioned 5' or 3' of the start of the first gene, or end of the last gene of the IgH C locus. This implies that the first or last gene of the IgH C locus is the coding region nearest to the NESSR.

The invention provides a genetically modified non-human mammal, or a genetically modified non-human mammalian cell as described herein having NESSR sites, which are preferably *lox*P sites, flanking the IgH C region genes, or inserted into the genes at each end of the IgH C region genes.

The invention provides a genetically modified non-human mammal, or a genetically modified non-human mammalian cell in which the endogenous IgH C genes are absent as described herein and a non-endogenous site specific recombination

(NESSR) site is present within the genome, preferably the non-endogenous site specific recombination site is a *lox*P recombination site.

It is preferred that the endogenous IgH C genes are deleted, but at least part of at least one endogenous IgH C enhancer sequence is retained. This has the advantage of improving expression of foreign genes when these are inserted and allows locus specific regulation of site-specifically introduced genes, (e.g. by using Cre-*lox*P insertion utilising the remaining *lox*P site in the deleted IgH C gene cluster). Retention of at least part of the endogenous J-C-intronic enhancer sequence and/or at least part of the α 3' enhancer sequence is particularly preferred.

In a genetically modified non-human mammal or cell of the invention, one or more endogenous Ig H variable region, D and/or J segment nucleic acid sequences may be present. It is particularly preferred that endogenous IgH variable region and D segment and J segment nucleic acid sequences are present.

A genetically modified non-human mammal or cell of the invention may comprise one or more selectable marker(s) integrated within the genome. A selectable marker may be positioned upstream of, or downstream of, a non-endogenous site specific recombination sequence. At least one selectable marker may be integrated within the genome upstream of, and/or downstream of, at least one non-endogenous site specific recombination sequence.

In a preferred embodiment, the invention provides a genetically modified non-human mammal, or a genetically modified non-human mammalian cell, having a selectable marker integrated upstream or downstream of the first and/or last endogenous IgH C gene and/or upstream or downstream of a *lox*P sequence.

The selectable marker is preferably one or more of: a neomycin resistance gene; a puromycin resistance gene; a hygromycin gene or a herpes simplex virus thymidine kinase gene.

The invention further provides a genetically modified non-human mammal, or a genetically modified non-human mammalian cell according as described herein characterised in that a different selectable marker is integrated at each end of the IgH C region.

Thus the invention also provides a non-human mammal or non-human mammalian cell, preferably a rodent cell, more preferably a mouse cell, most preferably a mouse embryonic stem cell, free from the endogenous immunoglobulin heavy chain locus and comprising one or more gene(s) encoding a selectable marker.

A genetically modified non-human mammal of the invention can be rodent, murine, ovine, porcine, equine, canine, feline or the like, but is preferably a rodent, more preferably murine, most preferably a mouse. A genetically modified non-human mammalian cell of the invention may be an embryonic stem cell or an oocyte; and is preferably a rodent, murine, ovine, porcine, equine, canine or feline cell, or the like, preferably a rodent cell, more preferably a murine cell, most preferably a mouse cell. Mice are particularly preferred as their immune repertoire is extensive and they are easy to handle and breed.

The present invention provides a mouse in which all 8 endogenous heavy chain constant region immunoglobulin genes (μ, δ, γ3, γ1, γ2a, γ2b, ε and α) are absent, or partially absent to the extent that they are non-functional, or in which genes δ, γ3, γ1, γ2a, γ2b and ε are absent and the flanking genes μ and α are partially absent to the extent that they are rendered non-functional, or in which genes μ, δ , γ3, γ1, γ2a, γ2b and ε are absent and α is partially absent to the extent that it is rendered non-functional, or in which δ, γ3, γ1, γ2a, γ2b, ε and α are absent and μ is partially absent to the extent that it is rendered non-functional.

By partially absent it is meant that the endogenous IgH constant region gene sequence has been deleted or disrupted to the extent that no functional endogenous IgH C gene product is encoded at the IgH C locus, i.e. that no functional endogenous IgH C gene product could be expressed from the locus.

The present invention further provides a non-human mammalian embryonic stem (ES) cell characterised in that the endogenous Ig heavy chain constant region genes are absent or partially absent. Preferably all of the endogenous IgH C region genes are absent; more preferably all the known endogenous IgH C genes are absent.

In a preferred embodiment the ES cell is a deletion mutant mouse embryonic stem cell in which all 8 endogenous heavy chain constant region immunoglobulin genes μ, δ, γ3, γ1, γ2a, γ2b, ε and α are absent or partially absent to the extent that they are non-functional, or in which genes δ , γ3, γ1, γ2a, γ2b and ε are absent and the flanking genes μ and α are partially absent to the extent that they are rendered non-functional, or in which genes μ, δ , γ3, γ1, γ2a, γ2b and ε are absent and α is partially absent to the extent that it is rendered non-functional, or in which genes δ, γ3, γ1, γ2a, γ2b, ε and α are absent and μ is partially absent to the extent that it is rendered non-functional.

The deletion mutant non-human mammal, preferably a rodent, more preferably a mouse, can be bred with a compatible non-human mammal that is able to express one or more functionally active IgH C genes, preferably one or more functionally active human IgH C genes, e.g. a deletion mutant mouse can be bred with a mouse capable of expressing one or more functionally active human IgH C genes. The heterozygous progeny (F1) of this cross can be inter-bred to produce heterozygous and homozygous progeny (F2) of a non-human mammal, preferably a mouse, that is able to express only foreign, preferably human, IgH C gene(s).

As shown in other Ig knock-out mouse strains expressing Ig transgenes, the presence of mouse C genes can result in the production of chimeric human (i.e. foreign) - mouse Ig chains by trans-switching or trans-splicing mechanisms that bring gene segments on different chromosomal locations together (reviewed in Brüggemann and Taussig, Curr. Opin. Biotechn., 8, 455-458, 1997). Thus, an advantage of having deleted the entire or essentially the entire endogenous IgH C gene region is that in F2 progeny, having and expressing an introduced e.g.,

exogenous IgH gene or locus, the endogenous IgH C gene locus cannot be re-activated to produce unconventional switch or splice products. Accordingly, an advantage of producing an animal with silenced endogenous Ig genes and introduced human Ig genes is that no mixed molecules (e.g. mouse IgH and human IgL) can be produced and that immunisation of that animal allows the production of specific fully human antibodies.

The invention provides a genetically modified non-human mammal derived from a genetically modified non-human mammal as described herein, or from a genetically modified non-human cell as described herein, and provides a genetically modified non-human cell derived from a genetically modified non-human mammal as described herein.

The invention provides a method for producing a genetically modified non-human cell comprising:

(a) (i) transfecting a non-human cell with a targeting construct for integration upstream of, or within the first IgH C gene of the IgH C locus, said targeting construct comprising a non-endogenous site specific recombination sequence and a selectable marker, selecting for a cell in which the selectable marker is present and screening said cell for integration of the recombination sequence, and,

(ii) transfecting a cell produced in (a)(i) with a targeting construct for integration downstream of, or within the last IgH C gene of the IgH C locus, said targeting construct comprising a selectable marker and a non-endogenous site-specific recombination sequence, selecting for a cell in which the selectable marker is present and screening said cell for integration of the recombination sequence; or,

(b) (i) transfecting a non-human cell with a targeting construct for integration downstream of, or within the last IgH C gene of the IgH C locus, said targeting construct comprising a non-endogenous site-specific recombination sequence and a selectable marker, selecting for a cell in which the selectable marker is present, and screening said cell for integration of the recombination sequence, and,

(ii) transfecting a cell produced in (b)(i) with a targeting construct for integration upstream of, or within the first IgH C gene of the IgH C locus, said targeting construct comprising a non-endogenous site-specific recombination sequence and a selectable marker, selecting for a cell in which the selectable marker is present, and screening said cell for integration of the recombination sequence; or,

(c) co-transfecting a non-human cell with a targeting construct for integration upstream of, or within the first IgH C gene of the IgH C locus and with a targeting construct for integration downstream of, or within the last IgH C gene of the IgH C locus, each of said targeting constructs comprising a non-endogenous site specific recombination sequence and each having a selectable marker, selecting for a cell in which the selectable marker(s) is/are present, and screening said cell for integration of the recombination sequence; and optionally,

(d) providing to a cell obtained in (a)(ii), (b)(ii) or (c) a recombinase active at the non-endogenous site-specific recombination sequence and screening for deletion events.

The recombinase in optional step (d) can be provided by an expression vector. In a preferred method, the non-endogenous site-specific recombination sequence is a *lox*P site and in optional step (d), the recombinase is a Cre recombinase.

It is preferred that the genetically modified non-human cell is an embryonic stem cell or an oocyte. The genetically modified non-human cell can be a rodent cell, more preferably a mouse cell.

Any suitable cloning vector may be used to generate the targeting construct, cloning strategies are described by Sambrook, Fritsch and Maniatis in Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989. Desirably, the targeting construct may carry one or more marker genes; suitable markers are known, especially suitable are those that allow for positive selection. Of particular interest is the use of the gene for neomycin phosphotransferase ("neo"), which confers resistance to G418, also suitable is the puromycin resistance gene ("puro")

or the hygromycin resistance gene; neomycin and/or puromycin resistance genes are preferred.

In the targeting construct, upstream and/or downstream from the target gene, may be a gene, which provides for identification of whether a homologous double crossover has occurred (negative selection). The Herpes simplex virus thymidine kinase gene (HSV-tk) may be used as a negative selection marker, since cells producing thymidine kinase may be killed by acyclovir or gancyclovir.

Once a targeting construct has been prepared and any undesirable sequences removed, the construct can be introduced into the target cell, for example an ES cell or an oocyte. Any convenient technique for introducing the DNA into the target cell may be employed. For conventional gene targeting (usually constructs up to 20 kb), DNA is most frequently introduced by electroporation (see Zou et al., Eur. J. Immunol., 25, 2154-62, 1995) whilst for secondary modifications, such as Cre-*lox*P mediated integration, electroporation can be used for integration of smaller constructs and other methods such as lipofection and yeast spheroplast/cell fusion for YACs (yeast artificial chromosomes) and calcium phosphate-mediated DNA transfer for chromosome-fragments or mammalian artificial chromosomes which would allow integration of several 100 kb up to the Mb range. Thus, electroporation is the preferred technique for introduction of small DNA fragments (up to 50 kb) into the target cell, the other methods listed are suitable and perhaps advantageous for the introduction of larger DNA sequences (>50 kb).

After transformation or transfection of the target cells, they may be selected by means of positive and/or negative markers. As previously indicated, positive markers such as neomycin and/or puromycin resistance genes can be used. Those cells with the desired phenotype may then be further analysed by restriction analysis, electrophoresis, Southern blot analysis, PCR, or the like.

PCR may also be used to detect the presence of homologous recombination. PCR primers can be used that are complementary to a sequence within the targeting construct, and complementary to a sequence outside the construct and at the target

locus. DNA molecules are obtained in the PCR reaction only when both the primers are able to bind to the complementary sequences, i.e. only if homologous recombination has occurred. Demonstrating the expected size fragments, verified by sequencing, supports the conclusion that homologous recombination has occurred.

While the presence of the marker gene in the genome indicates that integration has taken place, it is necessary to determine whether homologous integration has occurred. Methods for achieving this are known in the art, such as using DNA analysis by Southern blot hybridisation to establish the location of the integration. By employing probes for both the insert and the sequences at the 5' and 3' regions distant to the flanking region where homologous integration would occur, it can be shown that homologous targeting has been achieved. An advantage is that external probes adjacent to the targeting DNA and newly introduced restriction sites, for example by a selectable marker gene, can be used for identification of the targeted alteration

Thus screening, preferably by PCR, can be used to confirm integration of NESSR sites such as *lox*P sites in the correct position, on the same allele and in the correct (direct) orientation to allow gene removal by deletion. Screening using methods such as PCR can also be used to detect deletion events.

An embryonic stem cell as described herein, e.g. obtainable by the above method, can be used for the production of a genetically modified non-human mammal.

The above-described processes may be performed first to inactivate the constant heavy chain loci in an embryonic stem cell, the cells may then be injected into a host blastocysts and developed into a chimaeric animal. Suitable methods are described, for example, in Hogan et al., (Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994). Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbour Press NY). Chimaeric animals are bred to obtain heterozygous hosts. Then, by breeding of the heterozygous hosts, a homozygous host may be obtained.

Accordingly, the invention provides method for producing a genetically modified non-human mammal characterised in that an embryonic stem cell as described herein is introduced into a host blastocyst and developed into a chimaeric animal.

This can be achieved by a method characterised by:

(a) introducing a non-human mammal embryonic stem cell as described herein into a compatible non-human mammal blastocyst, and

(b) transplanting the blastocyst obtained in (a) into a compatible non-human mammalian foster mother to obtain a chimaeric non-human mammal, and optionally, screening for the selectable marker(s), and/or non-endogenous site specific recombination sequence(s), and/or for deletion of essentially all endogenous IgH C gene sequences.

A chimaeric non-human mammal produced by these methods can be bred to obtain heterozygous progeny. The heterozygous progeny can be inter-bred to obtain homozygous progeny.

The present invention also provides a method for producing a genetically modified non-human mammal according to the invention comprising:

(a) injecting a non-human mammalian ES cell clone having two integrated *lox*P sites as described herein into a non-human mammalian blastocyst,

(b) transplanting the blastocyst into a compatible non-human mammalian foster mother to obtain a progeny chimaeric non-human mammal,

(c) optionally screening for *lox*P,

(d) breeding the progeny to obtain a non-human mammal having two integrated *lox*P sites on the same allele,

(e) cross-breeding a non-human mammal having two integrated *lox*P sites with a compatible Cre expressing non-human mammal

(f) screening the progeny for deletion mutants, preferably by PCR, or

(g) generating genetically modified non-human mammals, e.g. mice, with two integrated *lox*P sites on one allele, or on one locus or on 2 separate alleles or loci, by cross breeding of mice derived either by transgenesis or the ES cell route,

(h) inter- or intra-allelic locus deletion upon Cre expression.

The invention also provides a method for producing a genetically modified non-human mammal characterised by cross-breeding a genetically modified non-human mammal homozygous for integration of a non-endogenous site-specific recombination sequence upstream of, or within the first IgH C gene of the IgH C locus with a compatible genetically modified non-human mammal homozygous for integration of a non-endogenous site-specific recombination sequence downstream, or within the last IgH C gene of the IgH C locus, to obtain heterozygous progeny and optionally interbreeding the heterozygous progeny to obtain progeny homozygous for both integrations.

The progeny homozygous for both integrations can be cross-bred with a compatible non-human mammal capable of expressing a recombinase active at the non-endogenous site specific recombination sequence to obtain progeny; with IgH C gene deletion, which optionally can be screened using suitable methods to detect IgH C gene deletion.

The non-endogenous site specific recombination sequence(s) can be *lox*P sites and the recombinase a Cre recombinase.

Progeny heterozygous or homozygous for *lox*P at both loci can be cross-bred with a compatible non-human mammal capable of expressing Cre recombinase to obtain a progeny non-human mammal that does not comprise a nucleic acid sequence which itself encodes any endogenous Ig heavy chain constant region locus polypeptide on one or both alleles.

A transgenic non-human mammal capable of expressing Cre recombinase may be prepared by microinjection of linearised Cre plasmid into male pronucleus of F1 non-human mammal embryos to produce Cre expressing non-human mammal strain.

Using methods of the invention described herein a genetically modified non-human mammal can be obtained that does not comprise a nucleic acid sequence which itself encodes any endogenous Ig heavy chain constant region polypeptide.

The invention provides a method for producing a genetically modified non-human mammal capable of expressing one or more exogenous genes, characterised by breeding a genetically modified non-human mammal that does not comprise a nucleic acid sequence which itself encodes any endogenous immunoglobulin heavy chain constant region locus polypeptide, with a compatible non-human mammal that encodes and is capable of expressing one or more exogenous gene(s), to obtain progeny heterozygous for the one or more exogenous gene(s), and optionally inter-breeding the heterozygous progeny to produce progeny homozygous for the one or more exogenous gene(s).

Thus the invention may be used to produce a non-human mammal, that is preferably a rodent, more preferably a mouse, that is capable of expressing foreign, preferably human immunoglobulin gene(s), by breeding the genetically modified non-human mammal, as defined herein that is unable to express functionally active (endogenous) IgH C genes, with a compatible non-human mammal, preferably a rodent, more preferably a mouse, that is able to express one or more functionally active IgH C genes. This enables inter-species gene/locus exchange to produce selected progeny (heterozygous or homozygous) with one or more functionally active exogenous, preferably human gene(s) of the desired traits in a background where the corresponding genes of the non-human mammal are silenced or removed.

A method is provided for producing a genetically modified non-human mammal or cell capable of expressing one or more exogenous gene(s) comprising introduction of one or more exogenous gene(s) into a non-human mammalian cell as described herein that does not comprise a nucleic acid sequence which itself encodes any endogenous immunoglobulin heavy chain constant region polypeptide. It is preferred that the non-human mammalian cell is an embryonic stem cell or an oocyte. When the non-human mammalian cell is an ES cell, it is preferred that the one or more exogenous gene(s) are introduced by transfection. When the non-

human mammal cell is an oocyte (egg cell) it is preferred that the one or more exogenous gene(s) are introduced by DNA micro-injection. Preferably the one or more exogenous gene(s) are inserted into the genome of the non-human mammal or cell, most preferably the one or more exogenous gene(s) are inserted into a non-endogenous site specific recombination sequence.

An alternative method for producing a genetically modified non-human mammal capable of expressing one or more exogenous gene(s) is provided, that comprises cross-breeding a non-human mammal that does not comprise a nucleic acid sequence which itself encodes any endogenous immunoglobulin heavy chain constant region polypeptide with a transgenic mammal having one or more exogenous gene(s) associated with or flanked by a non-endogenous site specific recombination sequence and having a recombinase active at the non-endogenous site specific recombination sequence to obtain progeny and optionally screening the progeny for insertion of the one or more exogenous gene(s).

In the above methods for producing of genetically modified non-human mammal or genetically modified non-human mammalian cell, capable of expressing one or more exogenous genes, it is preferred that the non-endogenous site specific recombination sequence is a *loxP* sequence and insertion is by Cre – *lox P* integration. The genetically modified non-human mammal is preferably a rodent, more preferably a mouse.

In order to provide for the production of xenogeneic (exogenous) binding proteins, (e.g. foreign antibody proteins) in a host, it is necessary that the host be competent to provide the necessary enzymes and other factors involved with the production of antibodies (e.g. the cellular recombination machinery), while lacking the endogenous genes for the expression of the heavy IgC sub-units of immunoglobulins and thus not able to express the remaining V, D and J segments after DNA rearrangement. Thus, those enzymes and other factors associated with germ line re-arrangement, splicing, somatic mutation, and the like are preferably functional in the host. However, a functional natural region comprising the various

exons associated with the production of endogenous immunoglobulin heavy chain constant regions will be absent/deleted in certain embodiments of the invention.

In a deletion and replacement strategy, the genetic material for insertion may be produced from a mammalian source, preferably a human source, or may be produced synthetically. The material may code for at least part of a known immunoglobulin or may be modified to code for at least part of an altered immunoglobulin. Suitable techniques for these processes are well known.

In the case of the deletion and replacement strategy, where the xenogeneic DNA insert is large, complete Ig loci (1-3 Mb) could be inserted. The use of the Cre-*lox*P replacement strategy would then allow locus removal and insertion of a different locus.

The exogenous gene or genes is preferably an Ig H gene or Ig H genes, more preferably an IgH C gene or IgH C genes. The exogenous genes or genes can be a human gene or human genes, preferably the exogenous genes are a human Ig heavy chain constant region genes, more preferably a human Ig heavy chain constant region locus, or a human Ig heavy chain locus, having V, D, J and/or C regions.

In the human, the immunoglobulin heavy chain locus is located on chromosome 14. In the 5'-3' direction of transcription, the locus comprises a large cluster of variable region genes (V_H), the diversity (D) region genes, followed by the joining (J_H) region genes and the constant (C_H) gene cluster. The size of the locus is estimated to be about 2,500 kilobases (kb). During B-cell development, discontinuous gene segments from the germ line IgH locus are juxtaposed by means of a physical rearrangement of the DNA. In order for a functional heavy chain Ig polypeptide to be produced, three discontinuous DNA segments , from the V_H, D, and J_H regions must be joined in a specific sequential fashion; V_H to DJ_H, generating the functional unit V_HDJ_H. Once a V_HDJ_H has been formed, specific heavy chains are produced following transcription of the Ig locus, utilising as a template the specific $V_HDJ_HC_H$ unit comprising exons and introns. There are two loci for Ig light chains, the κ locus on human chromosome 2 and the λ locus on human chromosome 22. The structure

of the IgL loci is similar to that of the IgH locus, except that the D region is not present. Following IgH rearrangement, rearrangement of a light chain locus is similarly accomplished by V_L and J_L joining of the κ or λ chain. The sizes of the λ and κ loci are each 1-3 Mb. Expression of rearranged IgH and an Igκ or Igλ light chain in a particular B-cell allows for the generation of antibody molecules.

The human Ig heavy chain locus V, D, J and/or C regions can be in germline configuration, or can be productively arranged. In germline configuration the exons are spaced by introns thus gene sequence must be rearranged for expression of the gene(s). When productively arranged, introns have been removed from the gene sequence and thus re-arrangement of gene sequence is not required for expression of the gene(s).

The invention provides a non-human mammal or cell capable of expressing one or more exogenous genes, obtainable by a method described herein and provides the use of a non-human mammal or cell in the production of exogenous, preferably human immunoglobulin.

It has been found that a transgenic non-human mammal can produce chimaeric or foreign immunoglobulin (derived from inserted genetic material) in response to an immunogen subsequently introduced to the transgenic non-human mammal. Accordingly, by introducing foreign, *e.g.* human, genetic material encoding for substantially the entire species-specific regions of an immunoglobulin it may be possible to stimulate the transgenic non-human mammal to produce foreign immunoglobulin to any antigen introduced to the animal. The transgenic animal could thus provide a highly useful, convenient and valuable source of human immunoglobulins to a large range of antigens. Furthermore, there is no interference due to endogenous IgH C polypeptide being simultaneously expressed.

Description of the Figures

Figure 1 illustrates the strategy for heavy chain immunoglobulin constant region gene removal. A *lox*P sequence upstream of a selectable marker gene (puromycin) was inserted at the most 5'C gene and another selectable marker gene (neomycin) upstream of a *lox*P sequence was inserted downstream of the last and most 3'Cα gene at the 3' enhancer. Upon Cre expression, this strategy resulted in the removal of a ~200kb region with all C genes.

Figure 2 illustrates the targeting construct for the 3' region, digests confirming correct assembly are provided on the left hand side.

Figure 3 illustrates a test Southern blot showing the germline (GL) fragments when hybridising with the 3' ext(ernal) probe indicated. Samples with homologous integration or knock-out (KO) will produce an additional ~10 kb SacI and ~4kb BamHI band in addition to a weaker germline band for the unmodified allele.

Figures 4 and 5 illustrate that following transfection of ZX3 (ZX3 is an in house designation, these murine embryonic stem cells were produced using methods described by Hogan, B., R. Beddington, F. Costantini, and E. Lacy. 1994 in Manipulating the mouse embryo, a laboratory manual. Cold Spring Harbor Laboratory Press), embryonic stem cells were selected and screened and from 601 ES cell clones, one targeting event, clone no. 355, was identified. Further Southern blot analysis of clone 355 is shown in Figure 5 left hand side.

Figure 6 illustrates the Cμ targeting construct and the sequence analysis of *lox*P in the 2 targeting constructs, which verified their correct orientation to allow Cre-mediated deletional removal of all C genes.

Figure 7 shows the scheme for targeted integration and deletion of the C gene cluster.

Figure 8 shows Southern blot analysis of germline transmission mice derived from ES clone 355 and their crossing to homozygosity. The ~4kb band indicates the targeting event, the ~6.5kb band is the germline band and ++ indicates targeted integration on both alleles.

Figure 9 shows the 355 ES cell clone that was used for further targeting with the Cμ targeting vector. External probes are indicated as A and B. This resulted in one targeting event, clone 212.

Figure 10 shows how integration was verified. Clone 212 carries two targeted integrations, upstream and downstream of the C gene cluster. Transfection of the ES cells with a Cre vector allowed deletion analysis and suggested that both targeting events are on one allele.

Figure 11 shows that clones after Cre transfection appear to produce a PCR band using primers 1 and 4 illustrated in Figure 7. However, one band indicating targeted integration, primers 1 and 2, remained. This may indicate mixed clones or that the Cre-*lox*P mediated removal is not achieved in all cells of the clones.

Figure 12 shows that, unexpectedly, about half the number of germline transmission mice had an allele carrying either the 5' or 3' targeting event, but not both events. As germline transmission was about 50% (half of the mice with the correct coat colour did not have the target modification) it seems that the two targeted integrations are on one allele but that cross-over frequently separated the two regions.

Figure 13 shows that analysis of a larger number of mice (including the mice shown in Figure 12) has resulted in identification of 7 mice that carry both targeting events.

Figure 14 shows the scheme for PCR analysis of IgHC knock out (deletion) mice.

Examples

Example 1: Preparation of the targeting construct for the 5' Cμ region

A λ phage library, obtained from E14 ES (embryonic stem) cell DNA (Sambrook, Frisch, Maniatis, Molecular Cloning, A laboratory manual, Cold Spring Harbor Laboratory Press, 1989), was hybridised with a 4.5 kb BamHI fragment comprising Cμ (Zou *et al.*, Int. Immunol. 13, 1489-1499, 2001) several positive clones were identified and mapped. Hybridisation methods are well documented in the literature and known by researchers skilled in the art.

A *lox*P site was added to the puromycin gene (Tucker *et al.*, Genes Dev., 10, 1008-1020, 1996) by PCR (forward primer oligo BamHI-*lox*P-puro: 5'TTTGGATCCATAACTTCGTATAATGTATGCTATACGAAGTTATCGACCT CGAAATTCTACCGGG3' (SEQ ID NO: 1) and reverse primer oligo-BclI-puro: 5' TTTGATCAGCTGATCTCGTTCTTCAGGC 3' (SEQ ID NO: 2) which allowed the retrieval of *lox*P-puro on a BamHI-BclI fragment).

The ~6.5 kb fragment from JH3/4 to Cμ1 and the ~5.5 kb fragment from Cμ2 to Cδ were linked with a *lox*P–puromycin resistance gene on a ~2 kb BamHI-BclI fragment (see Figure 6).

The targeting construct for Cμ was assembled by subcloning of BamHI-BglII fragments into pUC19 (Invitrogen). The 3' EcoRI site was replaced by NotI using partial digest and blunt end linker insertion. In the resulting 5' targeting construct for Cμ, a *lox*P sequence was inserted upstream of a selectable marker gene (puromycin) which was inserted at the Cμ gene.

Example 2: Preparation of the targeting construct for the α 3' region

The λ phage library (see above), obtained from E14 ES (embryonic stem) cell DNA

was hybridised with a 3.5 kb BglII fragment comprising the rat α 3'enhancer (Pettersson *et al.*, Nature, 344, 165-168, 1990) and several positive clones were identified and mapped.

The α 3'enhancer region on a ~9 kb SacI fragment was cloned into pUC19 and the internal EcoRV site was changed to SpeI by partial digest and blunt end linker insertion. This unique site allowed the integration of a ~1.3 kb Neomycin-*lox*P gene on a compatible NheI fragment (see Figure 2). A *lox*P site was added to the neomycin resistance gene (Stratagene, La Jolla, CA) by blunt end insertion of *lox*P from pGEM-30 (Gu, H., Y.-R. Zou, and K. Rajewsky. Cell, 73, 1155-1164, 1993) and by oligonucleotide insertion (Sauer, Mol. Cell. Biol., 7, 2087-2096, 1987) in the α3' targeting construct downstream of the neomycin gene.

Example 3: Confirming orientation of the *lox*P sites

Correct orientation of the *lox*P site in each targeting construct was verified by DNA sequencing (see Figure 6). The *loxP* sites must be in the same linear orientation to each other so that upon targeted insertion of both *loxP* sites Cre-mediated deletional removal can be obtained. Here, this allows the removal of both inserted selectable marker genes and the region between Cμ and the 3'α enhancer at the end of the C gene cluster.

Example 4: Preparation of ES cells

ZX3 ES cells, obtained from the 129 sv mouse strain were produced using methods described by Hogan, B., R. Beddington, F. Costantini, and E. Lacy. 1994 in Manipulating the mouse embryo, a laboratory manual. Cold Spring Harbor Laboratory Press. ZX3 is an in house designation for these murine embryonic stem cells derived by Zou Xiangang upon his 3rd attempt.

Examples 5: ES cell transfection and Southern hybridisation

The Cμ targeting construct was linearised using BamHI and NotI and the α 3'enhancer targeting construct was linearised with BglII and EcoRV (see Figure 3). For each construct separately, about 10 μg purified fragment (purification kit #28304, Qiagen, Crawley, West Sussex, UK) was mixed with ~10^7 ZX3 ES cells, obtained from the 129 sv mouse strain, and subjected to electroporation and selection as described (Zou *et al.*, Eur. J. Immunol., 25, 2154-2162, 1995; Zou *et al.*, Int. Immunol. 13, 1489-1499, 2001). DNA from G418 resistant (Neo) clones for the α 3'enhancer construct was prepared and analysed in Southern blots as described (Sambrook, Fritsch, Maniatis, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989). This allowed the identification of one correctly targeted clone (355) to be used for introduction of the Cμ construct and puromycin selection which resulted in one correctly targeted clone (212) identified with correct insertion into Cμ and the 3'α enhancer.

Hybridisation probes were a ~0.4 kb 3' external EcoRV-SacI fragment for the α 3'enhancer (see Figure 5) and 5' and 3' external probes for Cμ described in Zou *et al.*, Int. Immunol. 13, 1489-1499, 2001 (see Figures 9 and 10). These were the 5' external probe, a ~0.5 kb BamHI-BglII fragment, and a 335 bp 3' external probe, obtained by PCR using plasmid DNA with the following oligonucleotides: forward primer 5'AACCTGACATGTTCCTCC3' (SEQ ID NO: 3) and reverse primer 5'GGGATTAGCTGAGTGTGG3' (SEQ ID NO: 4). PCR conditions were 95°C 1 min, 58°C 1 min and 72°C 30 sec for 30 cycles.

Southern blots were carried out as above with the results given in Figures 3, 4 and 5 which shows the germline (GL) fragments when hybridising with the 3' ext(ernal) probe indicated. Samples with homologous integration or knock-out (KO) produced an additional ~10 kb SacI and ~4kb (3.8 kb) BamHI band in addition to a weaker germline band for the unmodified allele.

Figure 9 shows results from ES cell clone 355 which was used for further integration of the Cμ targeting vector. Southern blot analysis was carried out using probe B and probe A (not shown) which identified one targeting event, clone 212.

Verification of the two targeted integration events upstream (5' Cμ) and downstream (α 3') of the C gene cluster was confirmed in clone 212 by transient transfection of the ES cells with the Cre vector and deletion analysis by PCR using primer pair 1-4 (see Figure 7, or P1 and P4 see PCR methods) which showed deletion of the C gene cluster and suggested that targeted integration of the 2 constructs happened on one allele (see Figure 11).

Figure 10 shows how integration was verified by Southern blot analysis as described above. Clone 212 carries two targeted integrations, upstream and downstream of the C gene cluster. Transfection of the ES cells with the Cre vector allowed deletion which increased the resulting hybridisation band as expected.

Figure 11 shows that clones after Cre transfection appear to produce a PCR band using primers 1 and 4 illustrated in Figure 7. However, one band indicating targeted integration, primers 1 and 2, remained. This may indicate mixed clones or that the Cre-*lox*P mediated removal is not achieved in all cells of the clone. Note, as this is not seen in Southern blot hybridisations, Figure 10, is it likely to represent low level contamination picked up by very efficient PCR amplification.

Example 6: Generation of mice and breeding

ES cell clones with targeting events (355 for the single α 3'enhancer targeting event and 212 for the α 3' enhancer and 5' Cμ targeting events) were injected into BALB/c blastocysts, transplanted into (C57BL/6 x CBA)F1 foster mothers and chimaeric mice and germline transmission was obtained as described (Hogan, B., R. Beddington, F. Costantini, and E. Lacy. 1994. Manipulating the mouse embryo, a laboratory manual. Cold Spring Harbor Laboratory Press). The mice were further analysed by PCR as described below and were derived, bred and investigated in accordance with UK Home Office project licence regulations.

Examples 7: Southern blot analysis of germline transmission mice derived from ES clone 355

Southern blot analysis of germline transmission mice derived from ES clone 355 with the mice derived therefrom crossed to homozygosity was carried out using the 3' external E probe (C in Figure 7). The ~4kb band indicates the targeting event, the ~6.5kb band is the germline band and ++ indicates targeted integration on both alleles. The results of the Southern blot are provided in Figure 8.

For the derivation of transgenic mice expressing Cre-protein ubiquitously, the Cre plasmid pBS185 (GibcoBRL, Life Technologies, Paisley, UK) was linearised with ScaI and purified using a DNA purification kit (#28304, Qiagen, Crawley, West Sussex, UK). DNA was microinjected into the male pronucleus of F1 embryos (CBA x C57Bl/6) according to standard methods (Hogan, see above) and several founders were produced, two of which showed a high gene/locus deletion rate when crossed with *lox*P mice.

Figure 12 shows that, unexpectedly, about half the number of germline transmission mice obtained from clone 212 had one allele carrying either the 5' or 3' targeting event, but not both events. As germline transmission was about 50% (half of the mice with the correct coat colour did not have the site-specific modification) it seems that the two targeted integrations are on one allele but that cross-over frequently separated the two regions.

Analysis of a larger number of mice analysed by PCR identified up to now 7 mice which carried both targeting events (Figure 13). Breeding of these mice with Cre expressers showed locus deletion, resulting in production of a mouse with deletion of the IgH C gene cluster on one allele.

Example 8: PCR screening for IgH C knock out (deletion) mice

Primers

NAME (J. Coadwell)	NAME (L. Ren)	LENGTH	SEQUENCE (5' to 3')
P1 V00818f.pri	MIgHKO1F	27bp	AGAGCCCCCTGTCTGATAAGAATCTGG

P1 corresponds to SEQ ID NO: 5, this is a forward primer that binds to the μ region.

P2 Puromycinr.pri	MIgHKO2R	23bp	TGGATGTGGAATGTGTGCGAGGC

P2 corresponds to SEQ ID NO: 6, this is a reverse primer that binds to the μ region.

P3 Neomycinf.pri	MIgHKO3F	23bp	TGCTTTACGGTATCGCCGCTCCC

P3 corresponds to SEQ ID NO: 7, this is a forward primer that binds to the 3' enhancer region.

P4 X96607r.pri	MIgHKO4R	22bp	GAGTCCCCATCCCCAAGGCTGG

P4 corresponds to SEQ ID NO: 8, this is a reverse primer that binds to the 3' enhancer region.

PCR Methods

For each of the PCRs used for the mouse IgH knock-out screening the reactions were set up as follows:

Per 20 µl reaction;

10 x buffer	2.0 µl
2mM dNTPs	2.0 µ
20mM forward primer	0.8 µl
20mM reverse primer	0.8 µl
lab-prep. Taq	0.1 µl
diluted tail DNA	1.0 µl
water	13.3 µl

The forward and reverse primer pairs used for each PCR type were:

i. 212 PCR MIgHKO1F (SEQ ID NO: 5) and MIgHKO2R (SEQ ID NO: 6)
ii. 355 PCR MIgHKO3F (SEQ ID NO: 7) and MIgHKO4R (SEQ ID NO: 8)
iii. deletion PCR MIgHKO1F (SEQ ID NO: 5) and MIgHKO4R (SEQ ID NO: 8)

The 10x buffer used was:

500mM KCl
0.05% Tween20
100mM Tris-Cl pH 9.0
1.5mM $MgCl_2$.

The reactions were performed as follows:

5 minutes at 95 °C for 1 cycle, 30 seconds at 94 °C, then 45 seconds at the appropriate annealing temperature, followed by 1 minute at 72 °C for 30 cycles, then 10 minutes at 72 °C for 1 cycle. After which the reactions were held at 6 °C until they were analysed.

The annealing temperatures used for each reaction type were:

i. 212 PCR 62 °C

ii. 355 PCR 62 °C

iii. deletion PCR 66 °C.

Sequence Listing Information

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Claims:

1. A genetically modified non-human mammal or cell characterised in that it does not comprise a nucleic acid sequence which itself encodes any endogenous immunoglobulin heavy chain constant region locus polypeptide.

2. A genetically modified non-human mammal or cell according to claim 1 characterised in that it does not comprise a nucleic acid sequence which itself encodes any immunoglobulin heavy chain constant region (IgH C) polypeptide.

3. A genetically modified non-human mammal or cell according to claim 1 or claim 2 characterised in that all immunoglobulin heavy chain constant region gene sequences are absent or partially absent from the genome.

4. A genetically modified non-human mammal or cell according to any of the preceding claims, characterised in that it is obtainable or obtained by targeted deletion of essentially all endogenous IgH C gene sequences.

5. A genetically modified non-human mammal or cell according to any of the preceding claims characterised in that it is obtainable or obtained by Cre *lox*P recombination.

6. A genetically modified non-human mammal or cell according to any of the preceding claims characterised in that at least part of at least one IgH C gene enhancer sequence is present.

7. A genetically modified non-human mammal or cell according to any of the preceding claims characterised in that a non-endogenous site-specific recombination sequence is present within the genome.

8. A genetically modified non-human mammal or cell characterised by having a non-endogenous site-specific recombination sequence downstream of, or within the last gene of the IgH C locus.

9. A genetically modified non-human mammal or cell according to claim 8 characterised by having a further non-endogenous site specific recombination sequence upstream of, or within the first gene of the IgH C locus.

10. A genetically modified non-human mammal or cell according to any of the preceding claims characterised in that one or more endogenous Ig H variable region, D and/or J segment nucleic acid sequences are present.

11. A genetically modified non-human mammal or cell according to any of the preceding claims characterised in that one or more selectable marker(s) is present within the genome.

12. A genetically modified non-human mammal or cell according to claim 8 characterised in that at least one selectable marker is present upstream of, or downstream of the non-endogenous site specific recombination sequence.

13. A genetically modified non-human mammal or cell according to claim 9 characterised in that at least one selectable marker is integrated within the genome upstream of, and/or downstream of, at least one non-endogenous site specific recombination sequence.

14. A genetically modified non-human mammal or cell according to any of claims 11 to 13 characterised in that the selectable marker(s) is one or more selectable marker selected from a group comprising a neomycin resistance gene, a puromycin resistance gene, and a hygromycin resistance gene.

15. A genetically modified non-human mammal or cell according to any of claims 7 to 14 characterised in that the non-endogenous site-specific recombination sequence is a *lox*P site.

16. A genetically modified non-human mammal according to any of the preceding claims characterised in that it is a mouse.

17. A genetically modified non-human cell according to any of claims 1 to 15 characterised in that it is a mouse cell.

18. A genetically modified mouse according to claim 16, or a genetically modified mouse cell according to claim 17, characterised in that all eight endogenous IgH C genes μ, δ, γ3, γ1, γ2a, γ2b, ε and α are absent or partially absent.

19. A genetically modified non-human cell according to any of claims 1 to 15 or claim 17 or 18 characterised in that it is an embryonic stem cell.

20. A genetically modified non-human mammal derived from a genetically modified non-human mammal of any of claims 1 to 16 or claim 18.

21. A genetically modified non-human mammal derived from a genetically modified non-human cell of any of claims 1 to 15 or any of claims 17 to 19.

22. A genetically modified non-human cell derived from a genetically modified non-human mammal of any of claims 1 to 16 or claim 18.

23. A method for producing a genetically modified non-human cell comprising:

(a) (i) transfecting a non-human cell with a targeting construct for integration upstream of, or within the first IgH C gene of the IgH C locus, said targeting construct comprising a non-endogenous site specific recombination sequence and a selectable marker, selecting for a cell in which the selectable marker is present and screening said cell for integration of the recombination sequence, and,

(ii) transfecting a cell produced in (a)(i) with a targeting construct for integration downstream of, or within the last IgH C gene of the IgH C locus, said targeting construct comprising a selectable marker and a non-endogenous site-specific recombination sequence, selecting for a cell in

which the selectable marker is present and screening said cell for integration of the recombination sequence; or

(b) (i) transfecting a non-human cell with a targeting construct for integration downstream of, or within the last IgH C gene of the IgH C locus, said targeting construct comprising a non-endogenous site-specific recombination sequence and a selectable marker selecting for a cell in which the selectable marker is present, and screening said cell for integration of the recombination sequence, and

(ii) transfecting a cell produced in (b)(i) with a targeting construct for integration upstream of, or within the first IgH C gene of the IgH C locus, said targeting construct comprising a non-endogenous site-specific recombination sequence and a selectable marker, selecting for a cell in which the selectable marker is present, and screening said cell for integration of the recombination sequence; or

(c) co-transfecting a non-human cell with a targeting construct for integration upstream of, or within the first IgH C gene of the IgH C locus and with a targeting construct for integration downstream of, or within the last IgH C gene of the IgH C locus, each of said targeting constructs comprising a non-endogenous site specific recombination sequence and each having a selectable marker, selecting for a cell in which the selectable marker(s) is/are present, and screening said cell for integration of the recombination sequence; and optionally,

(d) providing to a cell obtained in (a)(ii), (b)(ii) or (c) a recombinase active at the non-endogenous site-specific recombination sequence and screening for deletion events.

24. A method according to claim 23 characterised in that the non-endogenous site-specific recombination sequence is a *lox*P site.

25. A method according to claim 24 characterised in that, in optional step (d), the recombinase is a Cre recombinase.

26. A method according to any of claims 23 to claim 25 characterised in that the recombinase is provided by an expression vector.

27. A method according to any of claims 23 to 26 characterised in that the genetically modified non-human cell is a mouse cell.

28.. A method according to any of claims 23 to 27 characterised in that the genetically modified non-human cell is an embryonic stem cell.

29. The use of an embryonic stem cell of claim 19 or a cell obtainable by a method of any of claims 23 to 28 for the production of a genetically modified non-human mammal.

30. A method for producing a genetically modified non-human mammal characterised in that an embryonic stem cell of claim 19 or obtainable by a method of claim 28 is introduced into a host blastocyst and developed into a chimaeric animal.

31. A method according to claim 30 characterised by:

(a) introducing a non-human mammal embryonic stem cell according to claim 19 or obtainable by a method of claim 28 into a compatible non-human mammal blastocyst, and

(b) transplanting the blastocyst obtained in (a) into a compatible non-human mammal foster mother to obtain a chimaeric non-human mammal, and optionally, screening for the selectable marker(s), and/or the non-endogenous site specific recombination sequence(s), and/or for deletion of essentially all endogenous IgH C gene sequences.

32. A method for producing a genetically modified non-human mammal characterised in that the chimaeric non-human mammal according to claim 30 or claim 31 is bred to obtain heterozygous progeny.

33. A method for producing a genetically modified non-human mammal characterised in that the heterozygous progeny of claim 32 is inter-bred to obtain homozygous progeny.

34. A method for producing a genetically modified non-human mammal characterised by cross-breeding a genetically modified non-human mammal homozygous for integration of a non-endogenous site-specific recombination sequence upstream of, or within the first IgH C gene of the IgH C locus with a compatible genetically modified non-human mammal homozygous for integration of a non-endogenous site-specific recombination sequence downstream, or within the last IgH C gene of the IgH C locus, to obtain heterozygous progeny and optionally interbreeding the heterozygous progeny to obtain progeny homozygous for both integrations.

35. A method according to claim 34 characterised by further comprising cross-breeding progeny homozygous for both integrations with a compatible non-human mammal capable of expressing a recombinase active at the non-endogenous site specific recombination sequence to obtain progeny; and optionally screening the progeny obtained for IgH C gene deletion.

36. A method according to claim 34 or claim 35 characterised in that the non-endogenous site specific recombination sequence(s) are *lox*P sites.

37. A method according to claim 36 characterised in that the recombinase is a Cre recombinase.

38. A method according to claim 36 characterised by further comprising cross-breeding progeny heterozygous or homozygous for *lox*P at both loci with a compatible non-human mammal capable of expressing Cre recombinase to obtain a progeny non-human mammal that does not comprise a nucleic acid sequence which itself encodes any endogenous Ig heavy chain constant region polypeptide on one or both alleles.

39. A genetically modified non-human mammal characterised in that it is obtainable or obtained by a method of claim 35 to claim 38 and does not comprise a nucleic acid sequence which itself encodes any endogenous Ig heavy chain constant region polypeptide.

40. A method for producing a genetically modified non-human mammal capable of expressing one or more exogenous genes, characterised by breeding a genetically modified non-human mammal according to claims 1 to 7 or claims 10 to 16 or claims 18 to 21 that does not comprise a nucleic acid sequence which itself encodes any endogenous immunoglobulin heavy chain constant region polypeptide, with a compatible non-human mammal that encodes and is capable of expressing one or more exogenous gene(s), to obtain progeny heterozygous for the one or more exogenous gene(s), and optionally inter-breeding the heterozygous progeny to produce progeny homozygous for the one or more exogenous gene(s).

41. A method for producing a genetically modified non-human mammal or cell capable of expressing one or more exogenous gene(s) characterised by comprising introduction of one or more exogenous gene(s) into a non-human mammalian cell according to claims 1 to 7 or claims 10 to 15 or claims 17 to 21 that does not comprise a nucleic acid sequence which itself encodes any endogenous immunoglobulin heavy chain constant region polypeptide.

42. A method according to claim 41 characterised in that the non-human mammalian cell is an embryonic stem cell.

43. A method according to claim 42, characterised in that the one or more exogenous gene(s) are introduced by transfection.

44. A method according to claim 41 characterised in that the non-human mammal cell is an oocyte (egg cell).

45. A method according to claim 44, characterised in that the one or more exogenous gene(s) are introduced by DNA micro-injection.

46. A method according to any of claims 41 to 45 characterised in that the one or more exogenous gene(s) are inserted into the genome of the non-human mammal or cell.

47. A method according to claim 46 characterised in that the one or more exogenous gene(s) are inserted into a non-endogenous site specific recombination sequence.

48. A method for producing a genetically modified non-human mammal capable of expressing one or more exogenous gene(s) characterised by cross-breeding a non-human mammal that does not comprise a nucleic acid sequence which itself encodes any endogenous immunoglobulin heavy chain constant region polypeptide with a transgenic mammal having one or more exogenous gene(s) associated with or flanked by a non-endogenous site specific recombination sequence and having a recombinase active at the non-endogenous site specific recombination sequence to obtain progeny and optionally screening the progeny for insertion of the one or more exogenous gene(s).

49. A method according to any of claims 46 to 48 characterised in that the non-endogenous site specific recombination sequence is a *loxP* sequence and insertion is by Cre – *lox P* integration.

50. A method according to any of claims 40 to 49 characterised in that the genetically modified non-human mammal is a mouse.

51. A method according to any of claims 40 to 50 characterised in that the exogenous gene or genes is an Ig H gene or Ig H genes.

52. A method according to claim 51 characterised in that the Ig H gene or genes is an IgH C gene or IgH C genes.

53. A method according to any of claims 40 to 52 characterised in that the exogenous genes or genes are a human gene or human genes.

54. A method according to any one of claims 40 to 53 characterised in that the exogenous genes are a human Ig heavy chain locus having V, D, J and/or C regions.

55. A method according to claim 54 wherein the human Ig heavy chain locus V, D, J and/or C regions are in germline configuration.

56. A method according to claim 54 wherein the human Ig heavy chain locus V, D, J and/or C regions are productively arranged.

57. A non-human mammal or cell obtainable by a method of any of claims 40 to 56.

58. The use of a non-human mammal or cell according to claim 57 in the production of human immunoglobulin.

Abstract

A genetically modified non-human mammal or cell characterised in that it does not comprise a nucleic acid sequence which itself encodes any endogenous immunoglobulin heavy chain constant region locus polypeptide.

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23

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Figure 1

CONSTANT REGION GENE KO

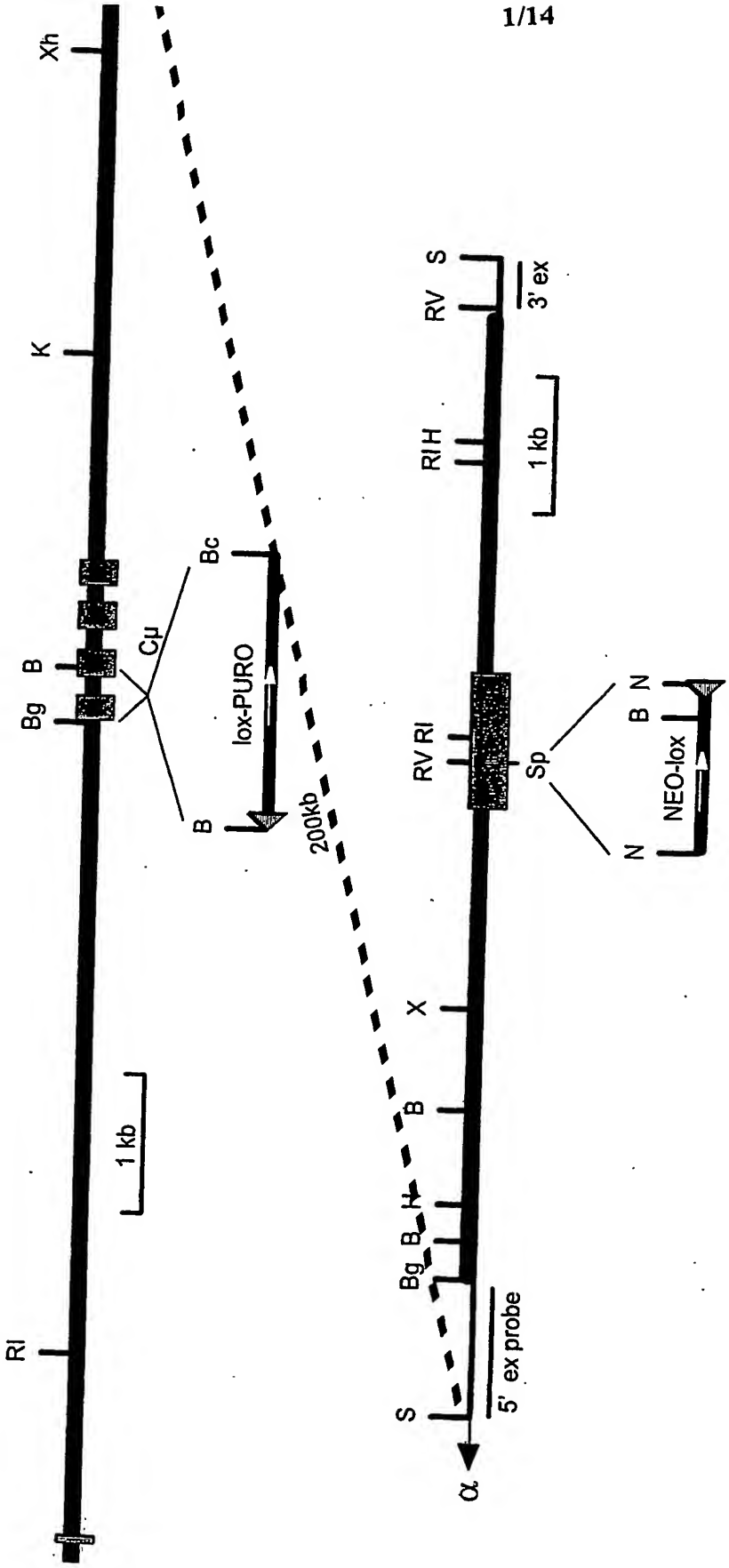
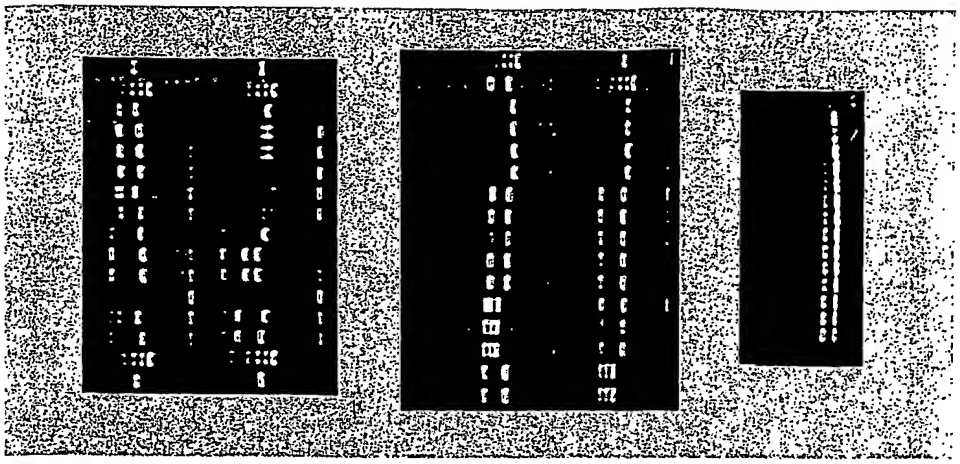
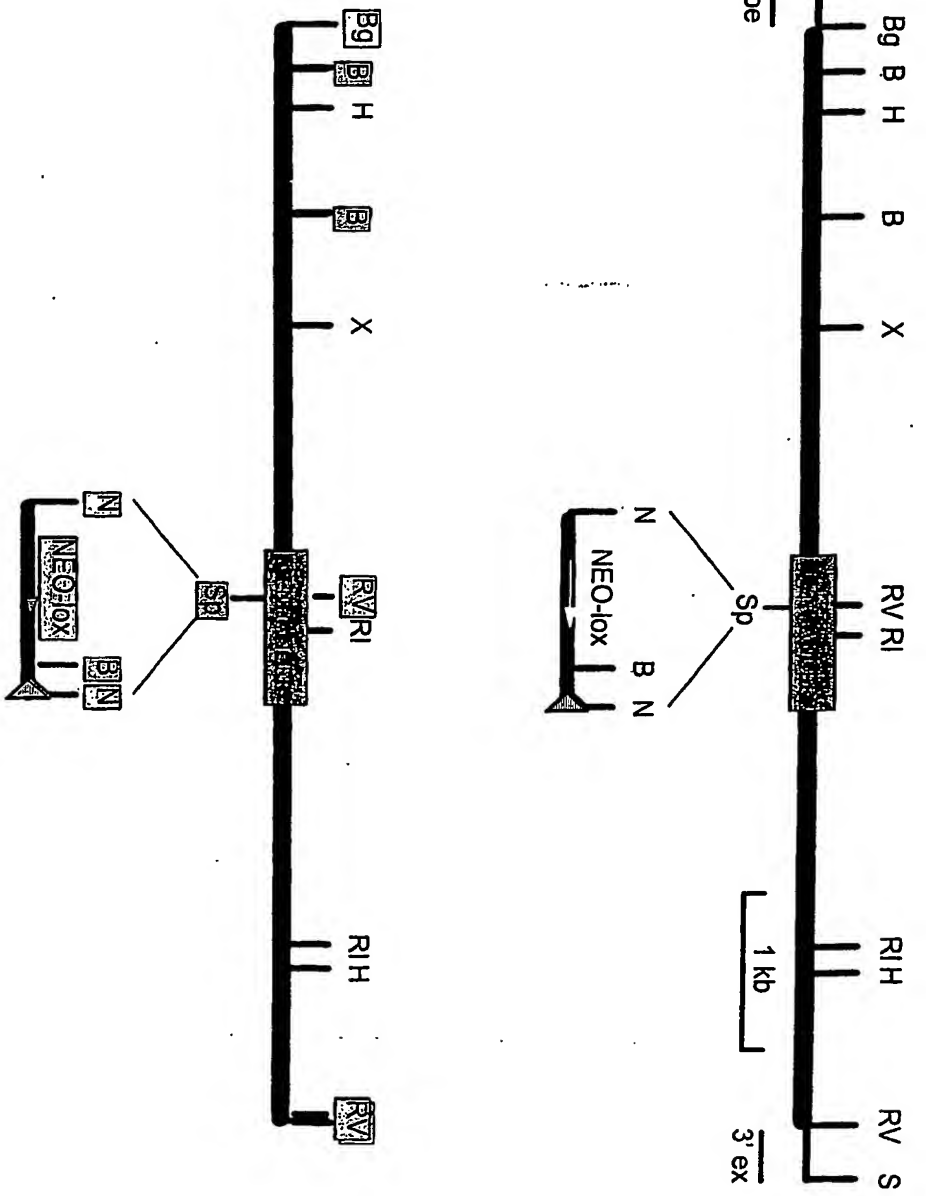


Figure 2

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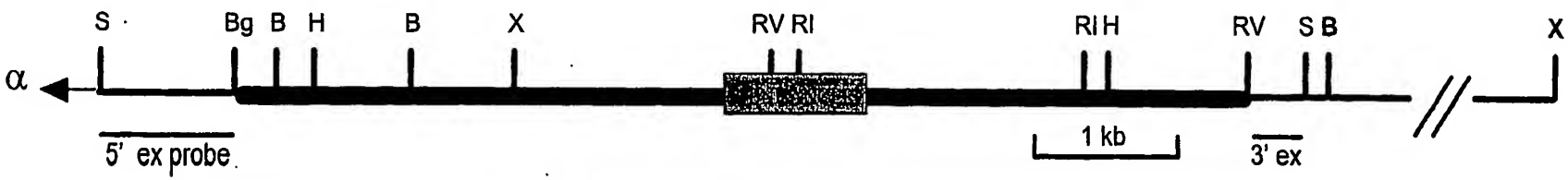
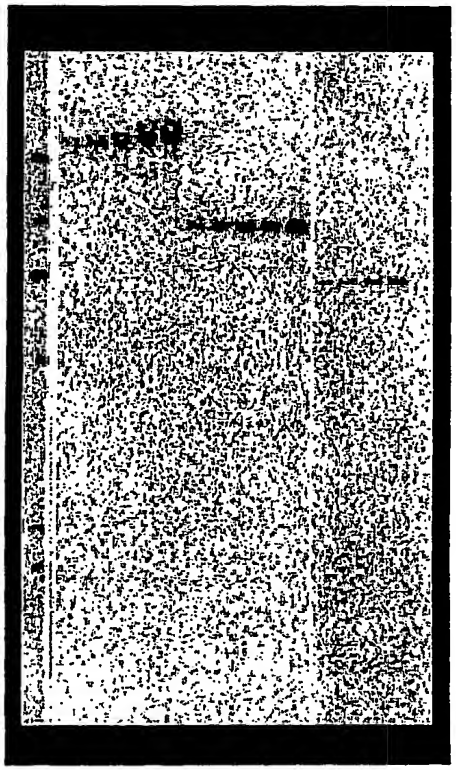
S
5' ex probe
Bg
B
H
B
X
RV
RI
Sp
N
NEO-lox
B
N
RI
H
1 kb
RV
3' ex
S
Bg
B
H
B
X
RV
RI
Sp
N
NEO-lox
B
N
RI
H
RV

Figure 3





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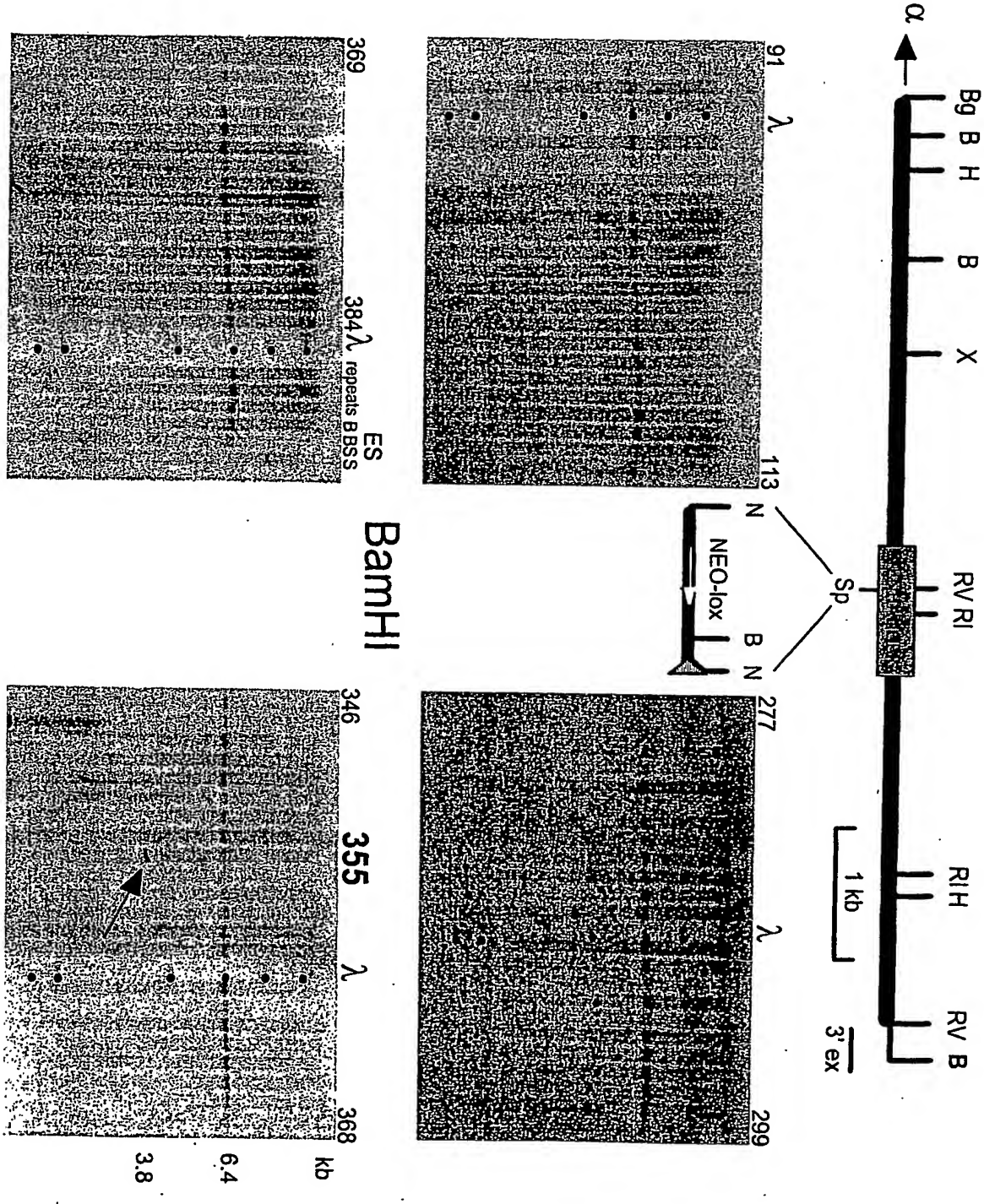
BamHI	6.5 kb	4 kb

3/14

Figure 4


α
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RV RI
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RV B
1 kb
3' ex
Sp
Sp
91
λ
113
N
NEO-lox
B N
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λ
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BamHI
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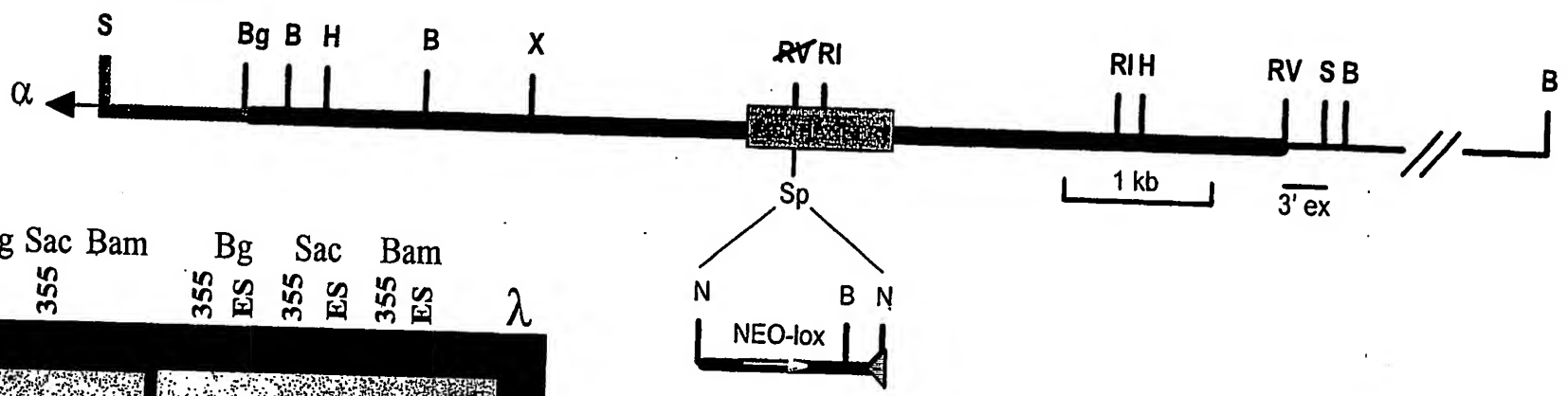
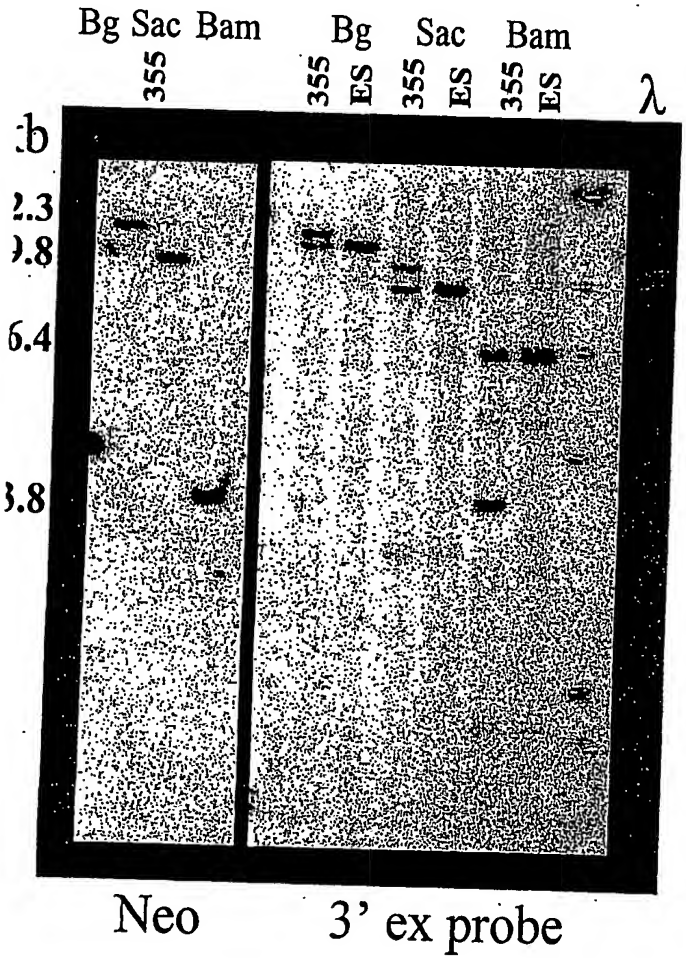
ES
BBSS
346
355
λ
368
kb
6.4
3.8

Figure 5





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0/217

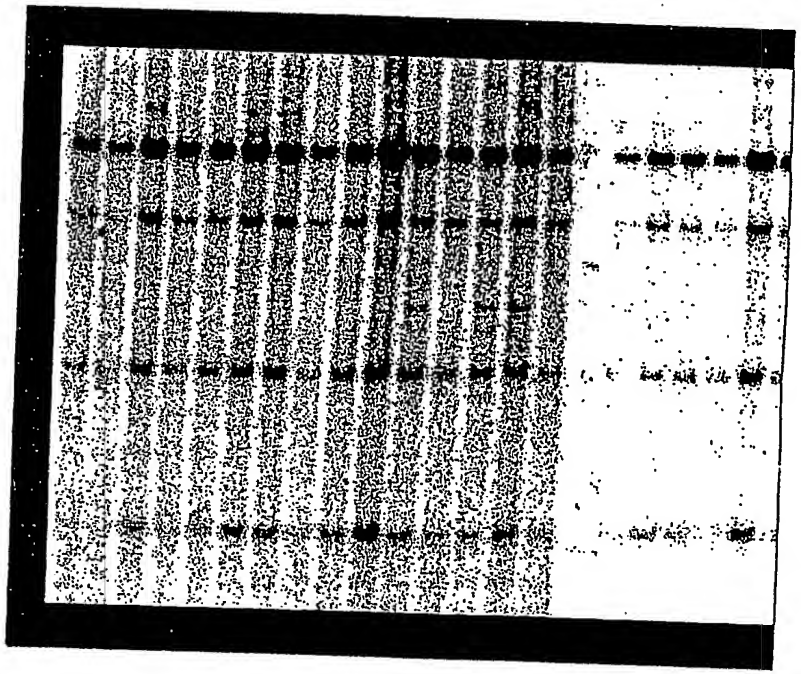
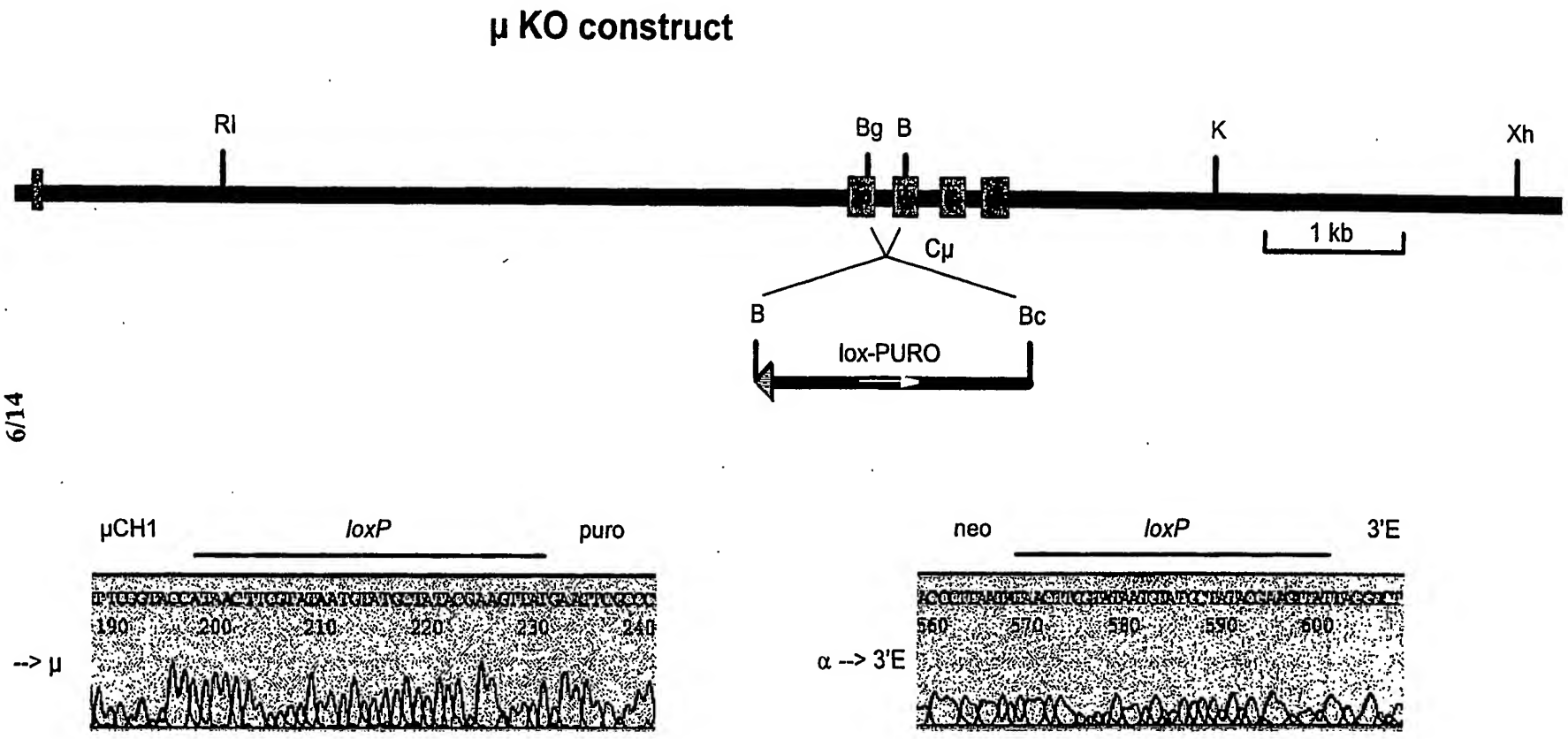


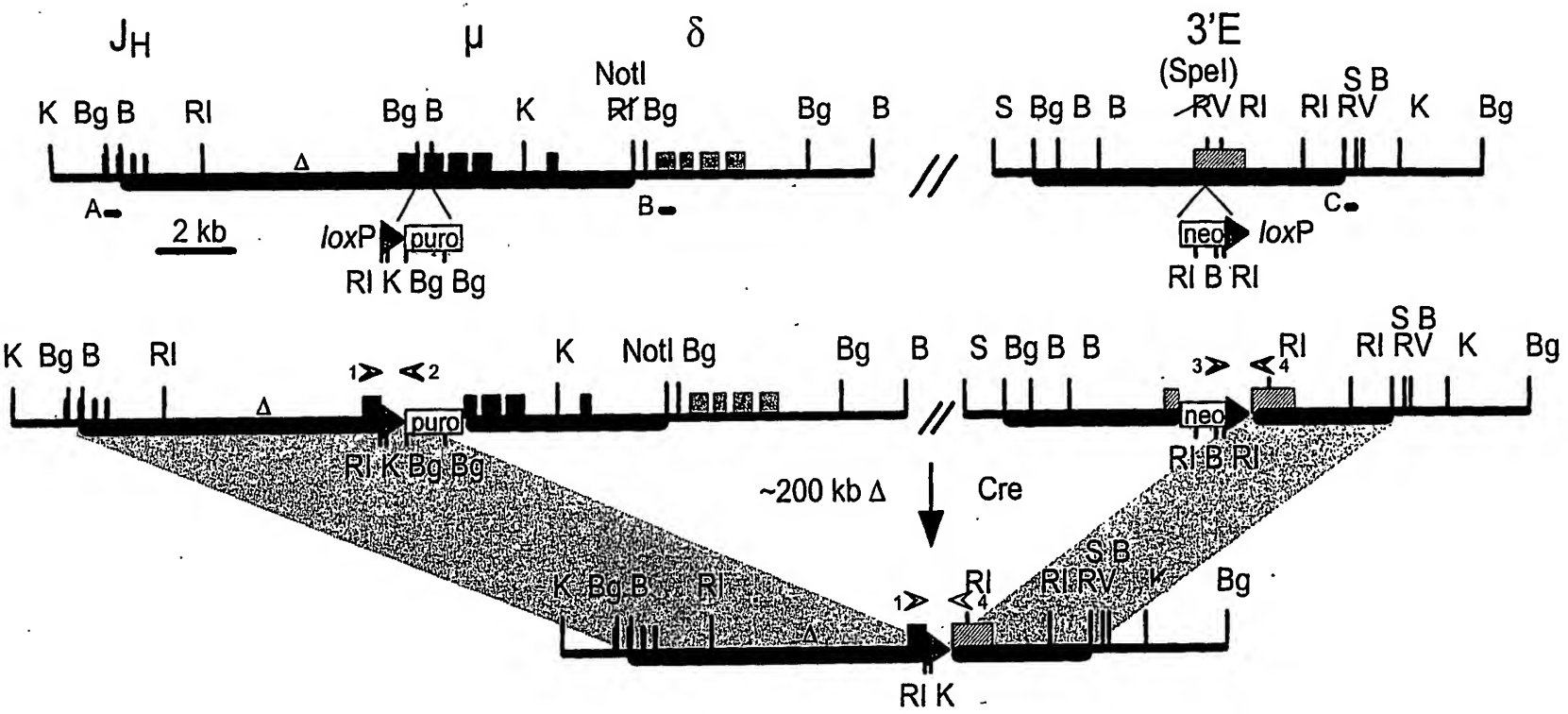
Figure 6


μ KO construct
RI
Bg B
K
Xh
1 kb
Cμ
B
Bc
lox-PURO
6/14
μCH1
loxP
puro
--> μ
190
200
210
220
230
240
neo
loxP
3'E
α --> 3'E
560
570
580
590
600

7/14

Figure 7

Targeting and deletion of the mouse IgH constant region genes



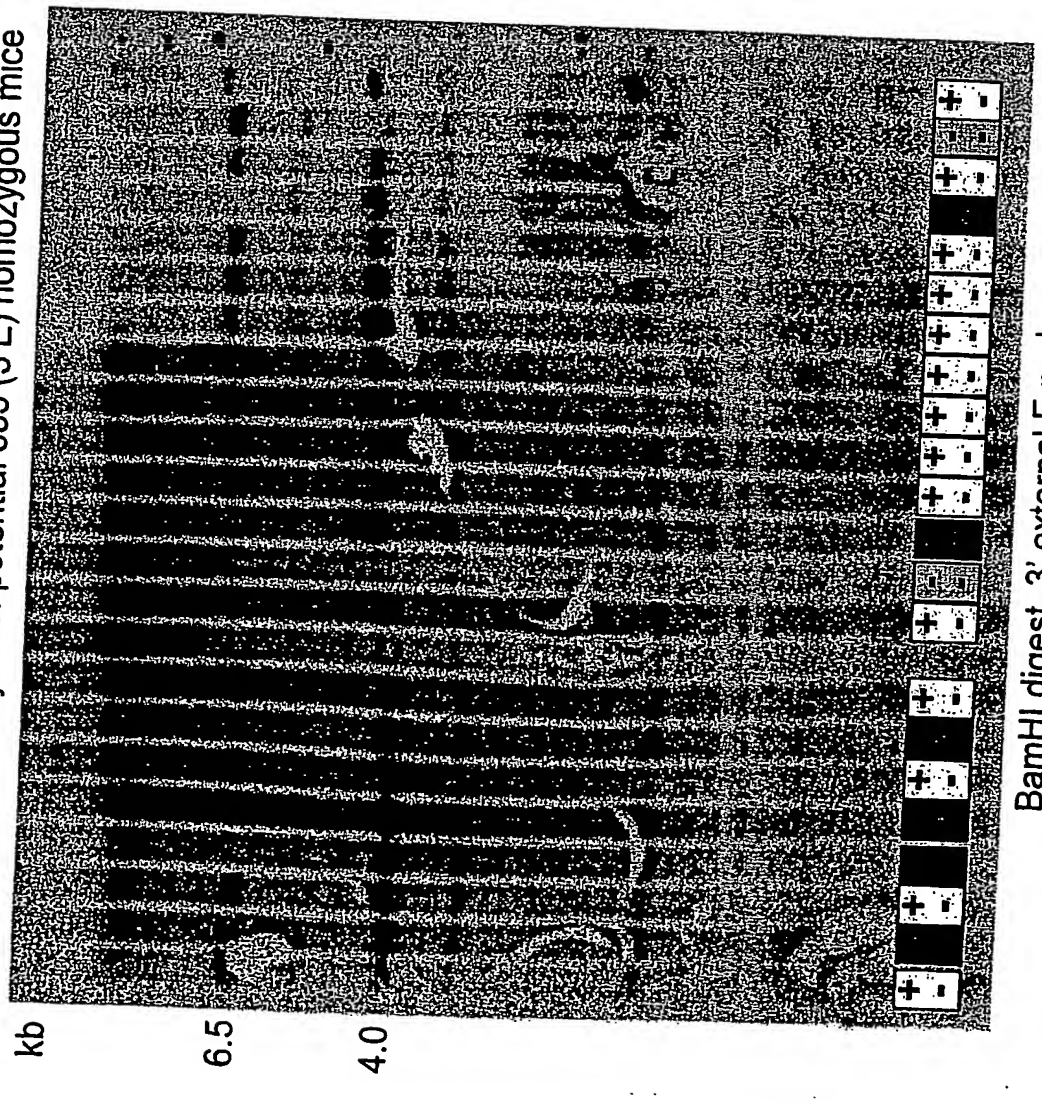
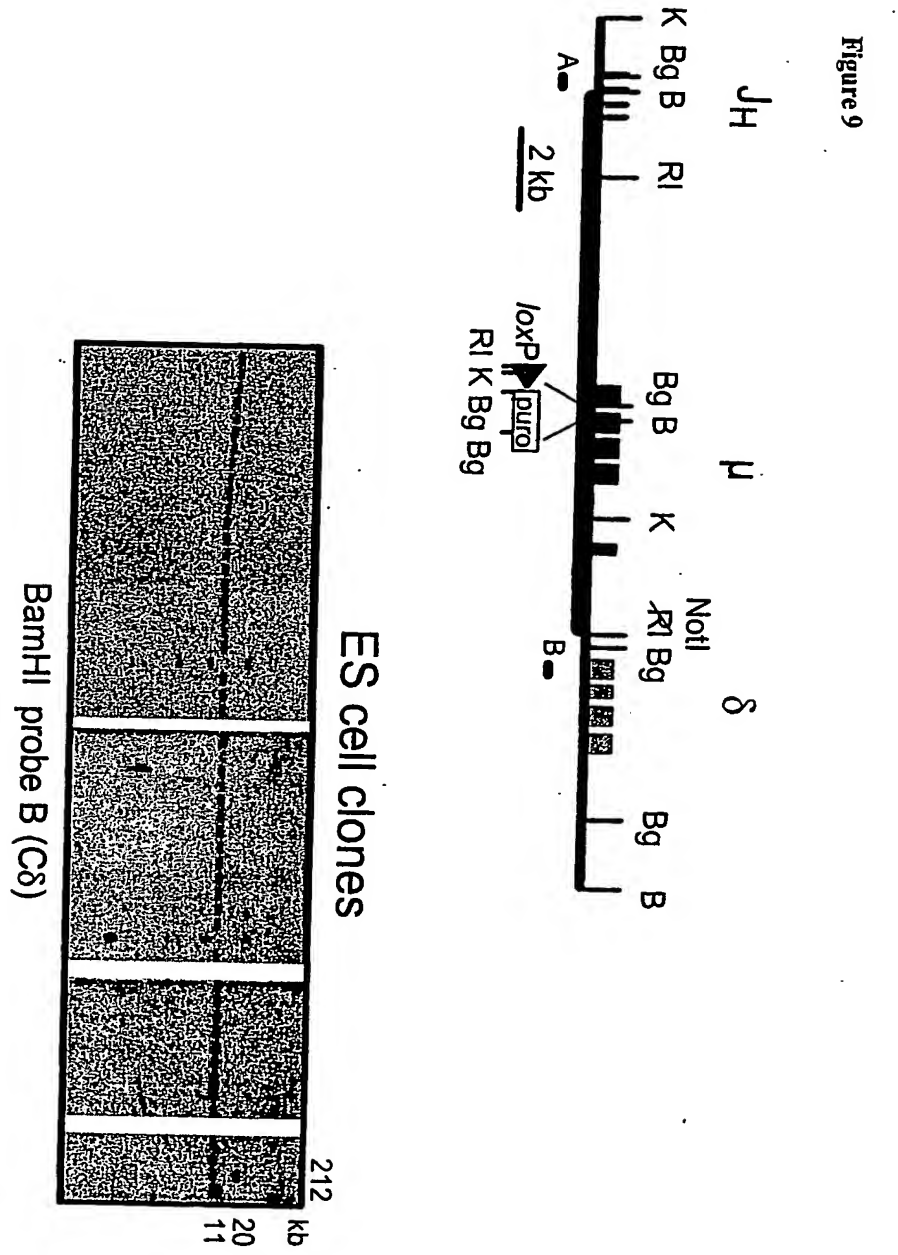

Southern blot analysis of potential 355 (3'E) homozygous mice
kb
6.5
4.0
BamHI digest, 3' external E probe

Figure 8

Figure 9


JH
μ
δ
K Bg B RI
A
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ES cell clones
BamHI probe B (Cδ)
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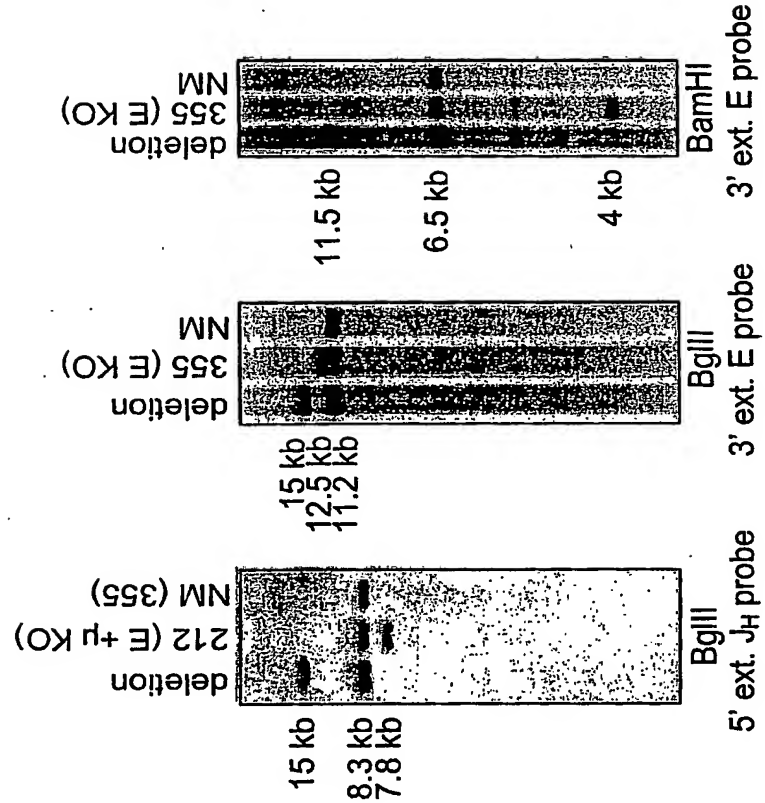
11
kb


ES cells +/- Cre
15 kb
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deletion
212 (E +μ KO)
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11.2 kb
deletion
355 (E KO)
NM
BglII
3' ext. E probe
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deletion
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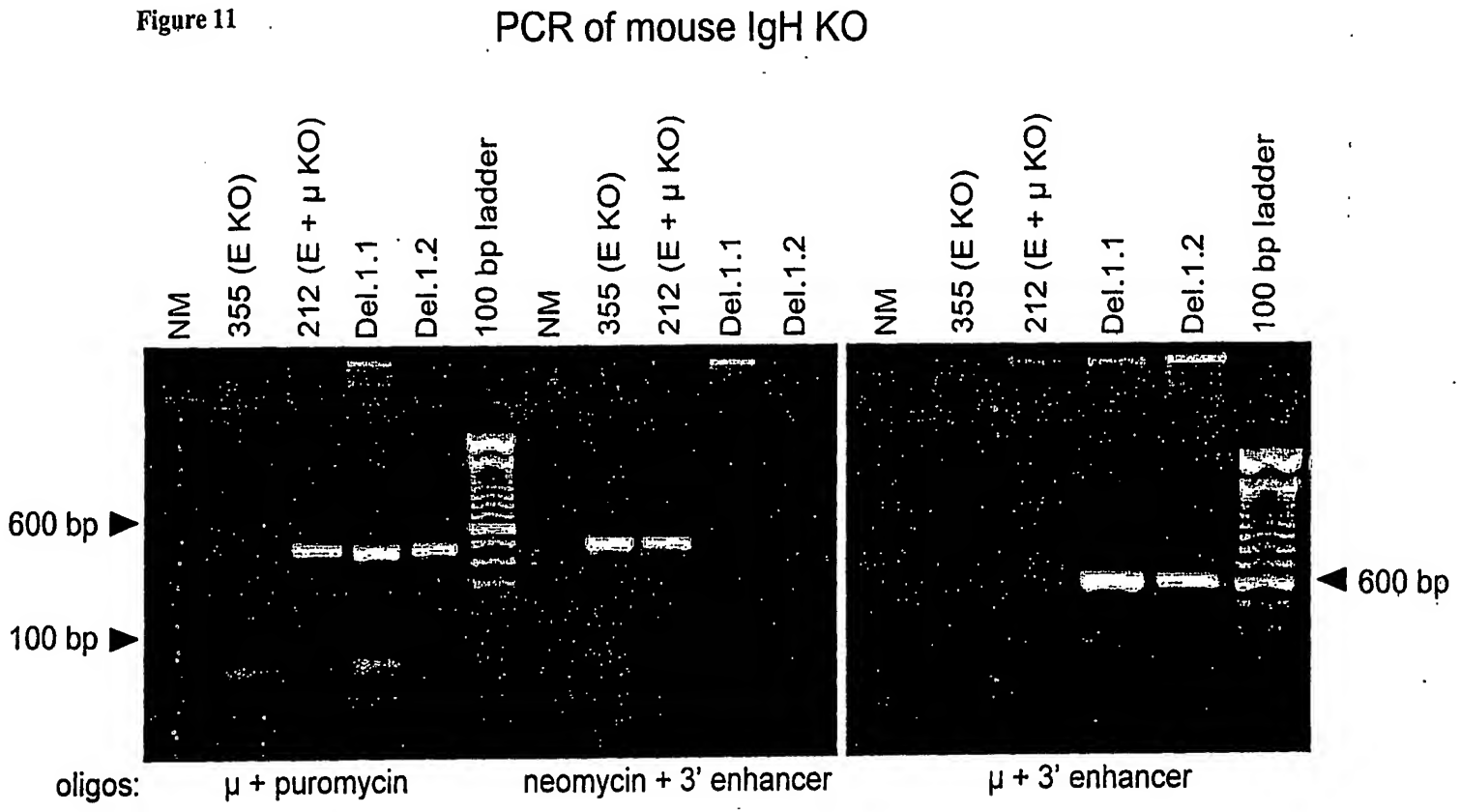
NM
BamHI
3' ext. E probe

Figure 10

; +/- Cre

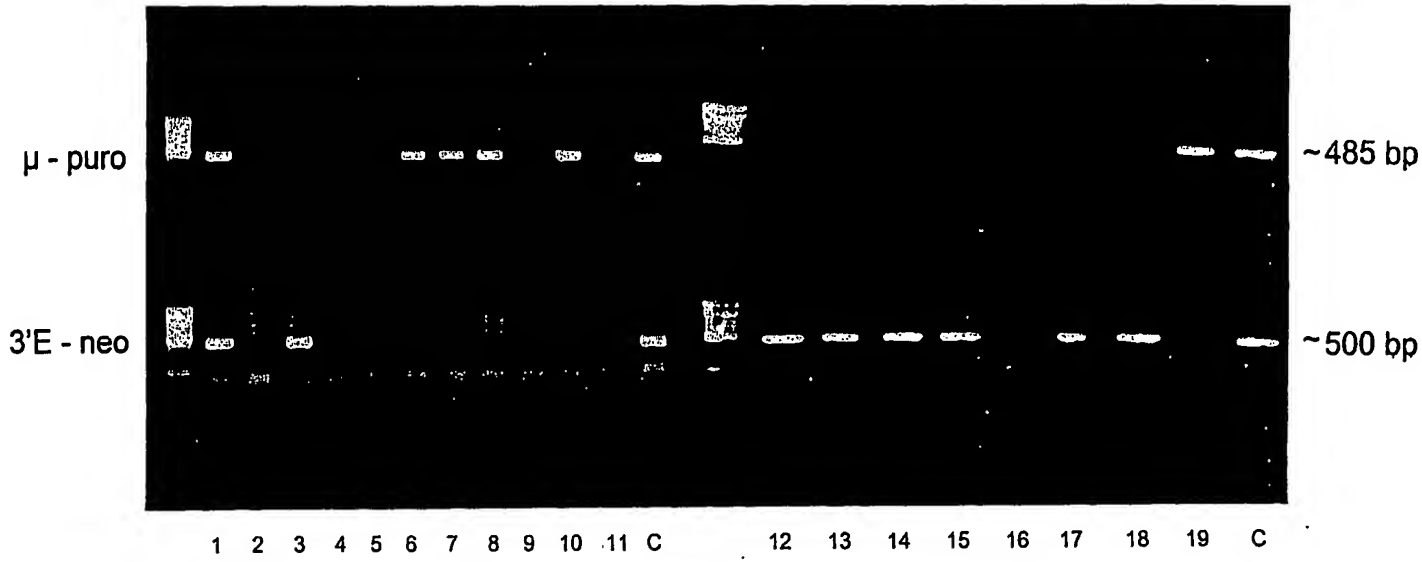
Figure 11



Beware: not all cells from the double targeted clone 212 carry a deleted C gene cluster after Cre transfection!

Figure 12

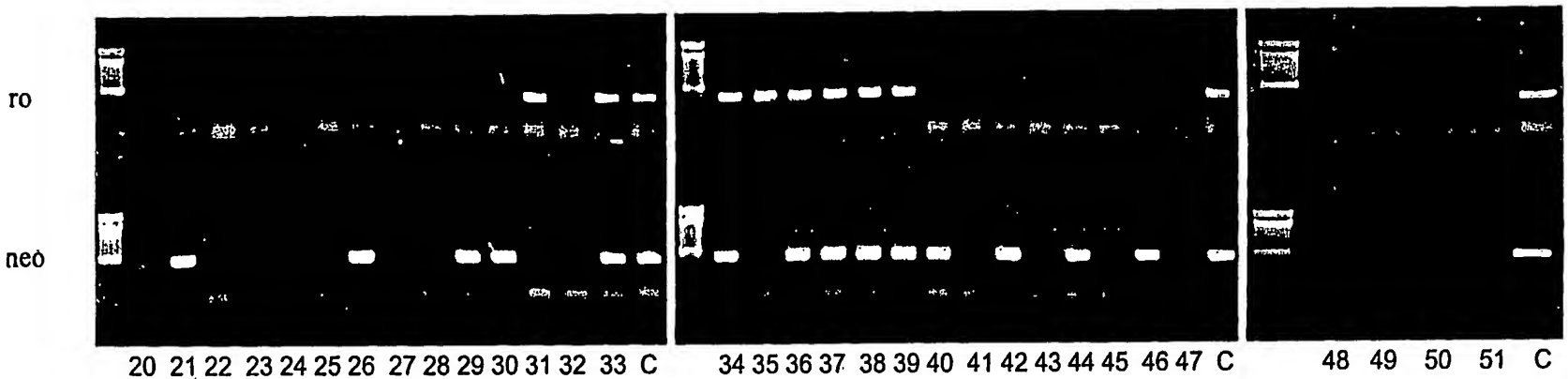
PCR analysis of potential (coat colour) germline transmission mice for both targeted integrations, 3'E and μ.



Result: from 19 mice 6 were negative, 7 positive for targeted integration in 3'E, 5 positive for targeted integration in μ and only 1 was positive for both targeting events.

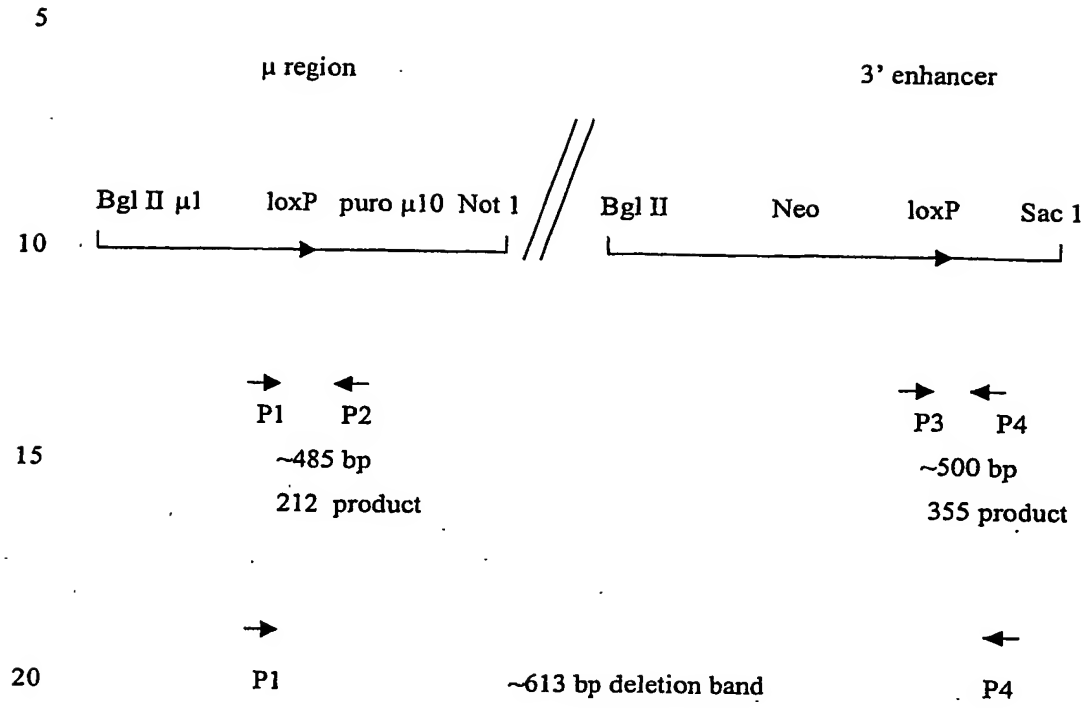
Figure 13

PCR analysis of potential (coat colour) germline transmission mice for both targeted integrations, 3'E and μ.



Result: from 51 mice 22 were negative, 14 positive for targeted integration in 3'E, 7 positive for targeted integration in μ and 7 were positive for both targeting events.

Figure 14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loxP
puro μ10
Not 1
Bgl II
Neo
loxP
Sac 1
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P2
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P3
P4
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355 product
P1
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P4